GENETICS

A periodical record of investigations bearing on heredity and variation

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L. J. Hradek

LEWIS JOHN STADLER

A TRULY great geneticist was lost in the death of Lewis J. Stadler on May 12, 1954. It is not often that the force of one man's intellect markedly influences the direction of scientific investigation but it is unquestionably true that Stadler's work had a salutary effect on the conclusions drawn from mutation studies. He was one of the foremost geneticists of his time. A masterful logician, a superb theoretician, he was also an able experimenter who conceived and executed genetical investigations of singular significance.

Stadler was born July 6, 1896, the second child of Henry Louis and Josephine Ehrman Stadler. His father was a successful St. Louis banker, and young Stadler was raised in a home where intellectual values were prized. He attended grade and high school in St. Louis. Stadler had evidenced some interest in agricultural pursuits as a youth and two summers during his high school years were spent on farms in Wisconsin and Missouri. Upon graduation from high school, he entered the College of Agriculture of the University of Missouri in 1913. After two years at Missouri he transferred to the University of Florida where he received the B.S. Agric. degree in 1917. He then returned to Missouri for graduate work in the Department of Field Crops but left after one year for Cornell University to work with Love and Emerson. Strange as it may seem to those who knew Stadler later, he did not make a favorable impression and was not encouraged to remain for further graduate work. Returning to the University of Missouri, he continued to work towards the Ph.D. degree, which was awarded him by this institution in 1922. In 1921 he was appointed Assistant Professor in the Department of Field Crops at the University of Missouri. Subsequently he was promoted to an Associate Professorship and finally to a full Professorship. Stadler remained at Missouri throughout his academic career save for a year spent as a National Research Fellow at Harvard and Cornell and for brief stays at the California Institute of Technology and at Yale University as a Visiting Professor. Under Stadler's leadership the genetics laboratory at Missouri became world famous, and many fellows came to work there. Not only did he bring renown to his school in genetics but the stimulating and intellectual atmosphere which he created made it possible for Missouri to attract able young people in related fields. He exerted an undoubted influence on all with whom he associated. An astute but sympathetic critic, he quickly perceived the essential points in an argument and often suggested critical experiments which would negate or prove an hypothesis. Although Stadler taught no formal courses, he was a superb teacher. Both articulate and logical, he had few peers in presenting difficult and recondite problems clearly and lucidly. He enjoyed engaging in informal conferences and discussions. No matter how exercised some participants might become, Stadler invariably presented his arguments quietly and persuasively. His poise and calmness never failed him.

The unusual quality of Stadler's intellect was evident in his early years, but he was an indifferent student in high school as well as in college. In fact it was not until he was well along in his graduate work and became interested in genetics by

reading Morgan's book "The Physical Basis of Heredity" that he found the area of biological research which fascinated him. Originally Stadler was not a geneticist but an agronomist concerned with field plot technique, and his early publications dealt with agronomic problems. It was not until 1925 that his first paper of a purely genetical nature appeared, but by this time he had launched an extensive series of genetic studies and a number of important papers shortly followed.

Throughout most of his scientific career Stadler devoted himself to the study of gene mutation, and it is in this area that his greatest contributions were made. Muller was the first to establish clearly the mutagenic effect of X-rays but Stadler independently and concomitantly was engaged in the study of the effects of short wave irradiation on the germinal material of plants. A few months after Muller's paper appeared, Stadler read a paper at the 1927 meetings of the AAAS in Nashville in which he corroborated Muller's conclusions. It seems somewhat ironical that, having been a pioneer in demonstrating the mutagenic effects of X- and gamma-rays, Stadler later showed that radiation-induced "gene mutations" in maize were not true gene transformations. The conclusion that X-ray induced mutations were extragenic alterations came only after a long and beautifully executed series of experiments in which he compared spontaneous and induced mutations at selected loci in maize and found that X-rays did not produce the kind of mutations arising spontaneously.

Large scale studies were made of the comparative effects of X-ray and ultraviolet irradiation, but much of the U. V. data have never been published in full, and some U. V. investigations were still under way at the time of Stadler's death. Although all the X-ray induced mutations were extragenic, it appeared that some of the U. V. induced mutations were indistinguishable from those arising spontaneously. When different regions of the U. V. spectrum were tested, it was found that the wave lengths characteristically absorbed by nucleic acids were the ones most effective in producing germinal changes.

Convinced that the kind of mutational changes giving rise to new gene forms of evolutionary importance were not produced by X-rays, Stadler spent his last years investigating spontaneous mutations at the *A* and *R* loci in maize. These studies are still in progress, but some significant results had been published. The *R* locus and that of *A*, as shown by Laughnan, are compound, consisting of genic entities of distinctive action but separable by crossing over. Unequal crossing over following oblique synapsis of these entities, which are homologous in terms of pairing affinity, leads to apparent or spurious gene mutation since chromatids result which are deficient or redundant for certain of the genic entities. In his work on the *R* locus Stadler noted that the kind of mutations detected experimentally were of relatively large effect, while the *R* alleles from diverse strains showed minute differences in the level of plant color expression. It was suggested that these subliminal mutations, presumably due to true gene mutation, whatever this may mean, are the ones responsible for the new gene forms of evolutionary significance and that the mutations with distinct differences usually identified in mutation experiments might all be due to extragenic alterations.

A characteristic feature of Stadler's work is the design of his experiments. He had

the ability to select well defined problems of a fundamental nature and to choose the experimental attack most likely to give unambiguous results. His published works, though relatively few considering the extent and comprehensiveness of his studies, were models of clarity and logical presentation. His arguments are presented with forensic finesse. He never published a trivial or inconsequential paper; all his contributions are major additions to genetic literature.

Many honors deservedly came to Stadler. He was a member of the National Academy of Sciences, the American Philosophical Society, and the American Academy of Arts and Sciences. He served as president of the Genetics Society of America (1938), and the American Society of Naturalists (1953). He was active in the Society of the Sigma Xi, first as president of the Missouri chapter (1931) and later as national president (1953–54). He was a member of numerous scientific bodies, editorial boards, and fellowship committees; to all these he rendered distinguished service.

In a day and age when the Ph.D. has come to stand for presumed mastery of a narrow area of specialization, Stadler was exceptional in the breadth of his culture and interests. He read widely and thoughtfully and conversed intelligently on many diverse subjects. He had a suave dignity and charm and a superb sense of humor. His philosophical outlook on the vagaries of life was with him to the end. Well aware of the nature of his illness and that death could not be far away, he accepted the inevitable with remarkable courage and fortitude. When death did come on May 12, 1954 in St. Louis, where he had gone for surgery, his body immediately was cremated, and no funeral services were held. All this was in accordance with his wishes. Quietly and unobtrusively he passed from this world, but his departure creates a void which will not soon be filled. The science of genetics has lost one of its great students, and this journal a distinguished member of its Editorial Board

M. M. RHOADES

STUDIES ON THE MECHANISM OF PATTERN FORMATION IN THE ELYTRA OF LADY BEETLES

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THE mechanism of the formation of various patterns in the wings of insects is a most intriguing problem from embryological, as well as cytological, genetical, and biochemical aspects.

In Lepidoptera, GOLDSCHMIDT (1932) has advanced a theory that the color pattern of wings was caused by the unequal rates of development of different parts. He has supposed that the color pattern would be changed as the rate of development was disturbed by a genic action.

In Coleoptera, GORTNER (1911) and TENENBAUM (1935) have speculated that the black pattern of the elytra of beetles was formed by the localization of melanin-chromogen. SUSSMAN (1949), WIGGLESWORTH (1950) and RICHARDS (1951) stated that the formation of the melanin pattern occurred according to the complex factors controlling the physiological status of epidermal cells developing in the region.

Harmonia axyridis Pallas, a species of lady beetle having four main variations with respect to pattern in the elytra, was used in our experiments. On the basis of the data on genetical analyses of HOSHINO (1936) and TAN (1946), it was assumed that these characters of pattern would be manifested by a set of genes belonging to multiple alleles.

Another type of lady beetle, *Coccinella bruckii* Mulsant, a closely related species, has no variation in the elytral pattern.

This present paper deals with the process of pattern formation, the analysis of the quantity of chromogen in the elytra before and after pigmentation, and also with the interaction between a few of the metabolic systems in epidermal cells of the elytra. From our data obtained by use of a chemical mutagen, nitrogen mustard, it is probable that the elytral pattern might be bestowed at the egg stage. The genic action in the manifestation of patterns has been also discussed from a viewpoint of biochemical genetics.

MATERIALS

Harmonia axyridis has a polymorphic elytral pattern which is due to a set of genes belonging to multiple alleles. In the natural populations of Japan, there are four main variations as follows: *conspicua* type (C-type), *spectabilis* type (S-type), *axyridis* type (A-type) and *succinea* type (s-type). The genes responsible for each character P^c, P^s, P^a and p show a dominance relationship designated as $P^c > P^s > P^a > p$. On the other hand, Coccinella sp. has no variation of the elytral pattern, but has a uniform color pattern of the type as shown in figure 1.

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EXPERIMENTAL METHODS AND RESULTS

Observations of the process of pigmentation

The soft and unpigmented elytra of adults immediately after emergence generally have a uniform color of yellowish white. Pigmentation of the various parts of these elytra begins at the same time as the hardening of the cuticle, and approximately two hours later, the pattern is formed completely as shown in figure 2. The formation of elytral pattern in the F_1 hybrid between homozygous C-type (P^c/P^c) and s-type (p/p) was observed. Fifty minutes after emergence, the pattern of s-type overlapped the pattern of C-type in the course of pigmentation, and only the latter pattern was recognizable at the end of pigmentation. The elytral pattern of the hybrid, however,

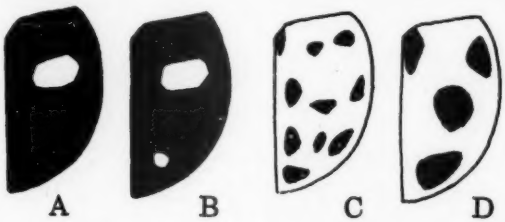

FIGURE 1.—The main variations of the elytral pattern in lady beetles. A: C-type. B: S-type. C: s-type. D: Coccinella sp.

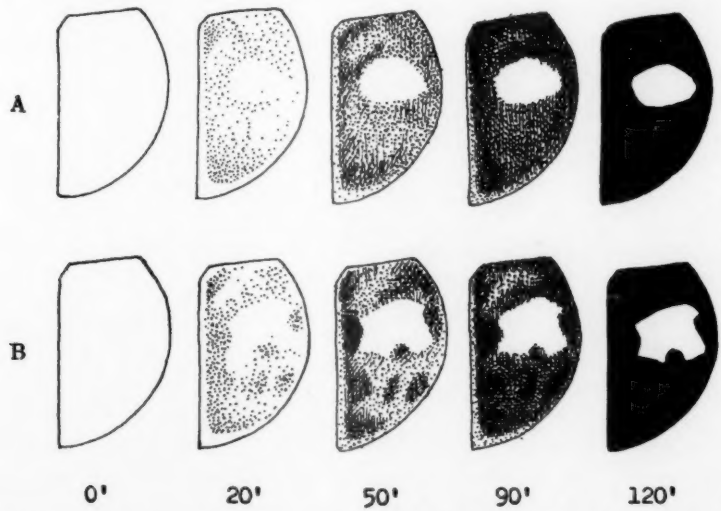

FIGURE 2.—The formation of the elytral pattern after emergence. A: C-type, homozygote P^c/P^c. B: C-type, heterozygote P^c/p.

generally had a number of depressions around the red colored region which showed no melanin.

TAN (1946) has found that the expression of the color pattern in the heterozygotes conformed to the rule of "mosaic dominance". This rule indicates that any portion of the elytra which has black pigment in the homozygote will also have black pigment in the heterozygotes in which the allele is present. The genic action of both P^c and p genes should be considered to have an equal bearing on the process of pigmentation.

Distribution and quantity of tyrosine, the substrate of melanin, in the elytra

Large quantities of tyrosine, the substrate of melanin, were extracted with 80 percent ethanol from the soft, unpigmented elytra which were pulverized immediately after emergence of the adult. The soft, unpigmented C-type elytra were separated into two parts by cutting them perpendicularly to the mid-line immediately posterior to the presumptive red portion. It was difficult to extract free tyrosine from the hard, black elytra. The tyrosine bound to protein could not be readily extracted with 80 percent ethanol, but was released by hydrolysis with 5N sodium hydroxide, at 110 $\sim$ 130°C, for 15 $\sim$ 20 hours. The quantity of tyrosine was measured by the following procedures: the elytra suspended in 80 percent ethanol were homogenized for six minutes by a Waring blender and centrifuged. The supernatant was evaporated to dryness and dissolved in buffer solution (pH 4.6, total vol. 5 ml). The precipitate was hydrolyzed with 5N NaOH, neutralized with acetic acid and adjusted to pH 4.6, and centrifuged. The precipitate was washed with buffer solution and the supernatant was combined with the washings of the precipitate giving a total volume of 10 ml. The two buffer solutions were adsorbed on the column (7 $\times$ 180 mm) of Amberlite IR-112 and eluted by M/10 citrate buffer (pH 4.6) containing 2 percent benzylalcohol (v/v).

TABLE 1

The quantity of tyrosine in the soft, unpigmented elytra (C-type)

	Dry weight of 100 elytra (mg)	Free tyrosine extracted with 80% ethanol (γ/mg)	Bound tyrosine released by hydrolysis with 5N NaOH 15 hrs, at 110°C (γ/mg)
Anterior region	46.0	6.8	30.0
Posterior region	32.0	5.9	30.3

TABLE 2

The quantity of tyrosine in the hard, black elytra

	Dry weight of elytra (mg)	Free tyrosine extracted with 80% ethanol (γ/mg)	Dry weight of elytra after extraction (mg)	Bound tyrosine released by hydrolysis with 5N NaOH: 15 hrs. at 110°C (γ/mg)
C-type	23.0	0.2	19.8	11.1
s-type	50.4	0.3	40.6	13.3
Coccinella	65.0	0.2	57.0	14.7

The effluent was separated by the automatic fraction collector constructed by T. SEKI (1953). To each fraction 5 ml of Na_2CO_3 5 percent solution and 1 ml of Folin phenol reagent were added, and after one hour, transmission of these colored fractions was measured by a photoelectric colorimeter. Tyrosine was generally found around the fifteenth fraction. The quantity of tyrosine in the elytra was calculated on the basis of the total color measurements (− log T) compared with the standards of tyrosine. Some of the histographs of the results of these experiments are presented in figure 3.

From the results shown in table 1, it was evident that the distribution of tyrosine in the soft unpigmented elytra was almost uniform and independent of the development of melanin pattern. Therefore, the general hypothesis, that the chromogen is localized at definite portions of the elytra, seems to be inadequate in this case. It was also

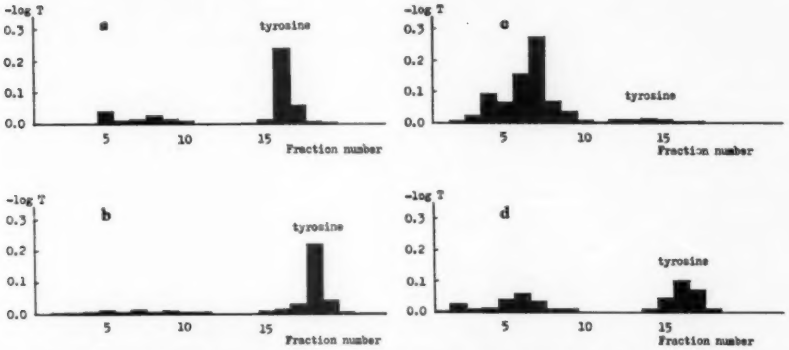

FIGURE 3.—Ion-exchange separation of tyrosine and other Folin positive substances (in C-type elytra). One fraction: 1.3 ml. a: Before pigmentation, extracted with 80% ethanol. b: Before pigmentation, hydrolyzed with 5N NaOH. c: After pigmentation, extracted with 80% ethanol. d: After pigmentation, hydrolyzed with 5N NaOH.

TABLE 3

Folin phenol reagent positive substances in various types of elytra (unit/mg)

Soft, unpigmented elytra	Extracted with 80% ethanol		Hydrolyzed with 5N NaOH: 15 hrs, at 110°C
	"A"	"B"	"C"
C-type	10.1	14.3	58.0
Hard, pigmented elytra	**Extracted with 80% ethanol**		**Hydrolyzed with 5N NaOH: 20 hrs, at 130°C**
	"A"	"B"	"C"
C-type	6.3	24.1	77.3
s-type	7.4	72.6	290.8
Coccinella	10.3	50.3	220.2

found that the content of the bound tyrosine in the light colored elytra was slightly higher than that in the black colored elytra (see table 2).

Substances colored by Folin phenol and uric acid reagents in the elytra and the body

In addition to tyrosine, several substances positive for the Folin phenol test were found in the fractions separated by use of a cation-exchange column. As shown in figure 3, the substances around the fourth and seventh fractions were designated as "A" and "B" substances, respectively; both were extracted with 80 percent ethanol.

TABLE 4

Folin phenol and uric acid reagent positive substances in the elytra and bodies of stored lady beetles

Elytra	Extracted with 80% ethanol (unit/mg)			Body	Extracted with 80% ethanol (unit/mg)		
	"A"	"B"	Uric acid in "B"		"A"	"B"	Uric acid in "B"
C-type	3.4	8.4	4.1	C-type	9.1	7.6	2.0
s-type	5.8	18.4	11.7	s-type	11.5	11.2	2.2

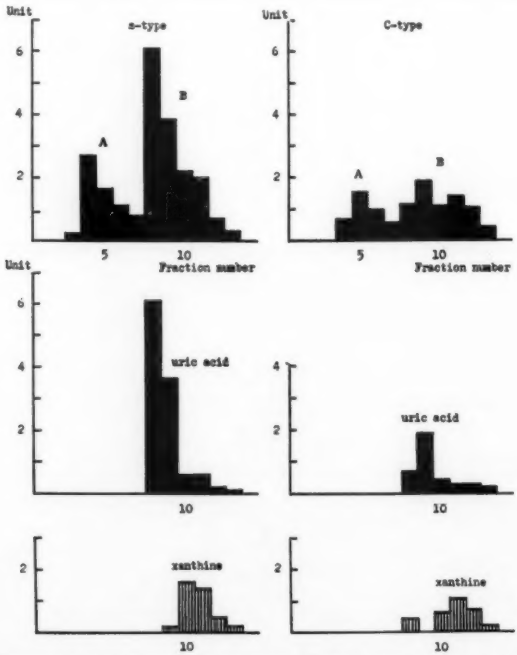

FIGURE 4.—Separation of "A" and "B" substances in the elytra of stored lady beetles by a cation-exchange column. Resin: IR-112, 200 mesh. Column size: 7 × 180 mm. Eluting solution: M/10 citrate buffer (pH 4.6) containing 2% benzylalcohol (v/v). One fraction: 1 ml.

The substance around the sixth fraction was called "C" substance and was hydrolyzed with sodium hydroxide. The content of these substances in various types of elytra is shown in table 3 by arbitrary units per microgram of dry weight of elytra. It was later found that the "B" substance was composed of uric acid and its precursor, xanthine. The quantities of uric acid and xanthine in "B" substance were separately obtained by determining the former by use of uric acid reagent.

The next experiment was carried out by using a number of previously stored lady beetles of C-type and s-type. The substances separately extracted with 80 percent ethanol from their elytra and bodies were treated by the same procedures as stated in the previous paragraph. The quantities of "A" and "B" and of uric acid in "B" substance were measured. The quantitative data and the histographs are shown in table 4 and figures 4 and 5.

From these results, a remarkable difference was noted between the "B" substances in the elytra and, to lesser extent, in the body of the two types. The elytra of s-type contained a larger quantity of uric acid than C-type, but in the body, the uric acid content of the two types was the same. "A" substance was presumably related to a polyphenol substance responsible for the tanning of cuticle. Further investigations will be undertaken on "A" and "C" substances.

Fluorescent substances in the elytra and body

Fluorescent substances were extracted from the elytra and bodies and purified as follows: the elytra were extracted four or five times with N/10 sodium hydroxide

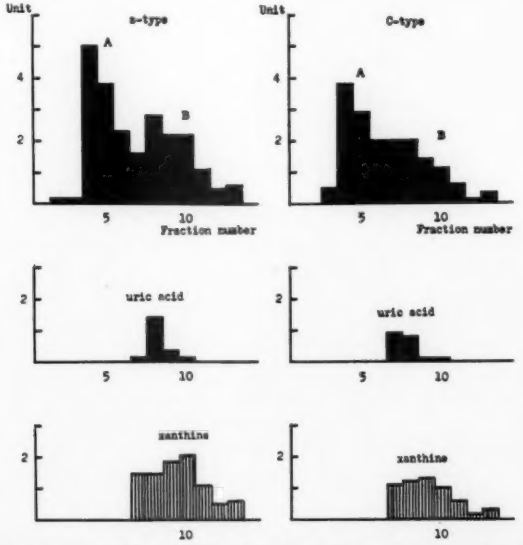

FIGURE 5.—Separation of "A" and "B" substances in the body of stored lady beetles by a cation-exchange column.

and the extracts were adjusted to pH 5 with acetic acid and adsorbed to Norit A. The fluorescent substances were eluted three or four times with a mixture of 50 percent ethanol and 5 percent ammonia (1:1) at 60°C, evaporated and then dissolved in 0.5 ml of N/10 sodium hydroxide. The aqueous solution thus obtained, as well as some synthesized pteridine derivatives and photodecomposed folic acid were paper chromatographized. Four percent citric acid solution, a mixture of n-butanol, glacial acetic acid and distilled water (4:1:1) and 3 percent ammonium chloride solution were used as developing solvents. Spots developed on the filter papers were detected under the ultraviolet rays. Several fluorescent spots were recognized and their Rf values were compared with those of the synthesized pteridines. The results are shown in table 5. Although several fluorescent substances were extracted from the elytra of Coccinella sp. and s-type, these substances, except for the spot no. 1 (table 5, part a), could not be detected in the extracts from the C-type elytra. The data of table 5 indicate that isoxanthopterin is contained in the red colored elytra in a larger quantity than other pteridines.

The fluorescent substance was readily extracted with acetone from the soft, unpigmented elytra immediately after emergence. The extracts were evaporated

TABLE 5

Rf values of the fluorescent substances extracted from the elytra and of several synthesized pteridines

Coccinella, s-type elytra				Synthesized pteridines		
Spot no.	Color	Rf.	Intensity*		Color	Rf.
			(a) Solvent: 4% citric acid solution: temp. 25°C			
1	purple	0.34	±	isoxanthopterin	purple	0.34
2	violet blue	0.53	+++	leucopterin	pale blue	0.34
3	violet blue	0.79	++	P-6-COOH†	sky blue	0.53
4	blue	0.94	+	P-6-CH_3	violet blue	0.53
			(b) Solvent: BuOH : AcOH : H_2O (4:1:1)			
1	?	0.09	+	leucopterin	pale blue	0.09
2	purple	0.21	++	P-6-COOH	sky blue	0.13
3	blue	0.47	+	isoxanthopterin	purple	0.21
				P-6-CH_2OH	sky blue	0.31
				P-6-CH_3	blue	0.47
			(c) Solvent: 3% NH_4Cl solution			
1	green	0.05	+	isoxanthopterin	purple	0.29
2	purple	0.29	+++	leucopterin	pale blue	0.32
3	?	0.47	±	P-6-CH_3	sky blue	0.47
4	sky blue	0.63	±	P-6-COOH	sky blue	0.47
5	yellow (?)	0.78	+	P-6-CH_2OH	sky blue	0.50

* Intensity: +++, strong; ++, moderate; +, weak; ±, very weak.

† P-6-COOH: 2-amino-4-hydroxy-6-carboxylic pteridine.
P-6-CH_3: 2-amino-4-hydroxy-6-methyl pteridine.
P-6-CH_2OH: 2-amino-4-hydroxy-6-ethyl pteridine.

and dissolved in 0.5 ml of distilled water. The aqueous solution was analyzed by paper chromatography. When developed by a mixture of n-butanol, glacial acetic acid and distilled water (4:1:1), it gave a sky-blue fluorescent spot with Rf about 0.1 under the ultraviolet rays. This fluorescent spot was segregated and eluted with distilled water from the paper chromatogram. The ultraviolet spectrum of the eluate showed the substance to be a type of pteridine having two maximums at 270 mμ and 360 mμ.

TABLE 6

Rf values of fluorescent substances from the elytra and bodies of lady beetles immediately after adult emergence

Spot no.	Color	Rf.	Identification
	(a) Extracted with 99% ethanol		
	(1) Solvent: BuOH : AcOH : H_2O (4:1:1)		
1	yellow	0.03	flavin (?)
2	sky blue	0.13	P-6-COOH
3	purple	0.22	isoxanthopterin
4	sky blue	0.38	P-6-CH_2OH
5	yellow	0.87	(?)
	(2) Solvent: 4% citric acid solution		
1	yellowish green	0.06	flavin (?)
2	?	0.09	(?)
3	?	0.23	(?)
4	purple	0.34	isoxanthopterin
5*	sky blue	0.55	P-6-COOH
	(b) Extracted with N/10 sodium hydroxide		
	(1) Solvent: BuOH : AcOH : H_2O (4:1:1)		
1*	blue (?)	0.09	P-6-COOH
2	purple	0.17	isoxanthopterin
3*	blue	0.36	P-6-CH_3
4	yellow	0.47	(?)
	(2) Solvent: 4% citric acid solution		
1	purple	0.32	isoxanthopterin
2*	blue	0.41	P-6-COOH
3	yellowish green	0.65	(?)
	(3) Solvent: 3% ammonium chloride solution		
1	purple	0.20	isoxanthopterin
2*	blue	0.40	P-6-COOH
3	yellowish green	0.43	(?)

* Fluorescent spots from the extracts of s-type lady beetle were larger in quantity than those from C-type lady beetle.

The elytra and the bodies of s- and C-type lady beetles immediately after adult emergence were extracted with acetone and the residues were further extracted with 99 percent ethanol and N/10 sodium hydroxide, respectively, in this order. Each of the extracts was treated as described at the beginning of this section and was analyzed by paper chromatography. The results on the paper chromatograms are shown in table 6.

From these results, it was considered that a larger quantity of the photo-decomposed pteridine of folic acid, e.g. P-6-COOH, was contained in the s-type than in the C-type lady beetles. In addition to this pteridine, isoxanthopterin and a certain riboflavin compound were also contained in the lady beetles when tested immediately after emergence.

The fluorescent substance, which was largely extracted with acetone from the soft, unpigmented elytra of Coccinella sp. was tested for its effect on melanogenesis. Since a large quantity of tyrosinase could not be obtained from the elytra of lady beetles, it was prepared from the body fluid of the mature silkworm (strain: Nichi 115, normal type). The acetone powder of the body fluid was suspended in about five parts of distilled water and stored for one hour in an ice box ($0 \sim 5°C$). The soluble enzyme was obtained by centrifuging the homogenate. This experiment was carried out by using a conventional Warburg apparatus, with the following composition of the vessel contents made up to a final volume of 2.44 ml.

Main compartment: enzyme solution 1.0 ml., 1 M phosphate buffer (pH 7.2) 0.24 ml and the extracted fluorescent substance; finally made up to 2.04 ml by the addition of distilled water.

Side arm: tyrosine or dopa; 1 micromol each in 0.2 ml distilled water.

Center well: 20% KOH 0.2 ml.

The above reaction mixture was incubated at 30.0°C. The results are shown in table 7.

The fluorescent substance partially inhibited the activity of tyrosinase and the rate of the inhibition was more remarkable when dopa, instead of tyrosine, was oxidized. The amounts of oxygen consumed between tyrosine and dopa were about twice as much in the presence of the fluorescent substance as in its absence. From these data, therefore, it is considered that the inhibitory action of the substance is exerted on the melanogenetic process below dopa, and that the oxidation of tyrosine to dopa is rather accelerated by its presence.

TABLE 7

Effects of the fluorescent substance extracted from the elytra of Coccinella sp. on the activity of tyrosinase

	O_2 uptake (cmm)				Inhibition (%)
	30 min.	80 min.	110 min.	140 min.	
Tyrosinase + tyrosine	21.5	58.5	61.0	61.8	
Tyrosinase + tyrosine + fluorescent sub.	32.3	51.7	54.0	52.8	13
Tyrosinase + dopa	19.5	50.5	53.4	55.1	
Tyrosinase + dopa + fluorescent sub.	24.5	38.2	40.9	39.8	27

Metals related to the color of elytra

The soft, unpigmented elytra of C-type were separated into two parts by cutting them perpendicularly to the mid-line and equal quantities of each part were burnt to ashes by dry oxidation and dissolved with a small amount of dilute HCl or HNO_3. The ash solutions thus obtained were examined by paper chromatography. A mixture of acetone n-butanol HCl (10:4:2) was employed as the solvent. The chromatogram was sprayed with suitable reagents such as 0.1 ~ 0.5 percent rubeanic acid dissolved in 98 percent ethanol, 1 percent potassium ethyl-xanthogenate dissolved in water, and kinds and amounts of metals were detected. The results are shown in figure 6. From the size of these spots, it was recognized that a large quantity of molybdenum was already present in the anterior part of the elytra, before hardening and darkening.

Then, the hard, pigmented elytra of C-type were divided along the lower margin of the red colored pattern and equal amounts of each part were subjected to wet oxidation with conc. HNO_3, H_2SO_4 and 60 percent perchloric acid. After the pH of the ash solutions was adjusted to pH 9.0 with ammonium citrate, the amounts of metals, e.g. molybdenum, copper and cobalt, were measured by the modified method of PARKS, HOOD, HURWITZ and ELLIS (1943).

From the data in table 8, it is evident that different amounts of molybdenum and cobalt are involved in each part after darkening and hardening of the cuticle. In view of these data on the different contents and distribution of the metals, it is

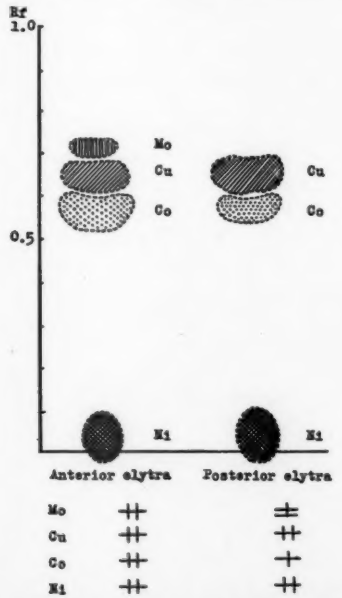

FIGURE 6.—Schematic representation of the paper-chromatographic patterns of metals from the soft, unpigmented elytra of C-type. Solvent: acetone n-butanol HCl (10 : 4 : 2).

assumed that the biological significance of these metals might be concerned with a preferential localization of the metallic enzymes in the elytra, e.g. molybdenum as a flavoprotein complex in the red colored portions of the elytra and copper as a tyrosinase distributed over the elytra in the process of hardening and pigmentation.

Effect of nitrogen mustard on the formation of elytral patterns

The eggs of lady beetles were smeared with nitrogen mustard which is known to be a mutagenic agent. Eggs, laid by the F_1 hybrids between the homozygous S-type

TABLE 8

The amounts of metals, molybdenum, copper and cobalt contained in the elytra of C-type

Type of metal	Anterior region γ/100 mg	Posterior region γ/100 mg
Mo	394	3
Co	79	45
Cu	87	92

TABLE 9

Action of nitrogen mustard at the various stages of development of lady beetles

Conc. of N.M. (%)	Time of smearing	No. of treated eggs	No. of hatched larvae	No. of adults	Percentage of hatched adults
1.0	immediately after laying	48	0	—	
	24 hrs after lay	48	0	—	
	48 hrs after lay	31	0	—	
	before pupation	102	102	1	1.0
0.2	immediately after laying	23	0	—	
	24 hrs after lay	43	0	—	
	48 hrs after lay	184	146	0	
0.1	48 hrs after lay	25	25	5	20.0
0.05	48 hrs after lay	48	48	14	29.2
Control (untreated)		74	74	33	44.6

TABLE 10

Occurrence of an unexpected elytral pattern in lady beetles following treatment with nitrogen mustard

Conc. of N.M. (%)		Homozygous S-type (P^s/P^s)	Heterozygous S-type (P^s/p)	Homozygous s-type (p/p)	Unexpected C-type
0.1	♀	0	1	1	0
	♂	2	1	0	0
0.05	♀	0	5	1	2
	♂	1	5	0	0
Control	♀	3	8	4	0
	♂	4	10	4	0

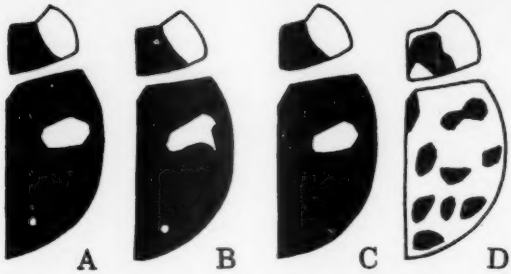

FIGURE 7.—The elytral and thorax pattern of the lady beetles in the experiment using nitrogen mustard. A: homozygous S-type (P^s/P^s). B: heterozygous S-type (P^s/p). C: unexpected C-type caused by the effect of nitrogen mustard. D: homozygous s-type (p/p).

and s-type of lady beetles, were used in this experiment. The deleterious action of nitrogen mustard at the various stages of development was observed as shown in table 9. All the eggs smeared with 0.2 percent nitrogen mustard died either at the egg or larval stage, but with 0.1 and 0.05 percent nitrogen mustard, some adults developed from the treated eggs. As shown in table 10 and figure 7, among these adult lady beetles, two individuals having an unexpected elytral pattern (C-type) were noted. The lady beetles with the unexpected pattern were observed to have the same pattern on their thorax and the anterior red colored pattern of their elytra as in the homozygous S-type lady beetle. Based on the evidence that the unexpected C-type pattern, possibly due to a somatic mutation from the S-type pattern, was obtained by the mutagenic effect of nitrogen mustard, it was assumed that the elytral pattern of an adult might be affected at the egg stage or approximately 48 hours after oviposition.

DISCUSSION AND CONCLUSION

PRYOR (1940) postulated that the cuticle of insects could be formed by the reaction between cuticle protein and oxidation products of phenol to yield sclerotin. A similar concept was extended by FRAENKEL and RUDALL (1947) who found that the amount of substrate, free tyrosine, increased in the body fluid of blowfly larvae before the hardening of pupal cuticle. RICHARDS (1951) speculated that tyrosine would be oxidized in the integument of arthropods as follows; tyrosine → dopa → lactic deriv. → propionic deriv. → acetic deriv. → benzoic deriv. He also assumed that the formation of the brown and black pigment patterns could be ascribed to the participation of tyrosinase at least partially in this process, and that a local melanization would occur by the localization of the substrate, while the enzyme was distributed uniformly over the cuticle. The speculation that pigment patterns could be formed by the localization of chromogen, has been favored by a series of experiments of GORTNER (1911), TENENBAUM (1935) and DANNEEL (1943) etc. This concept was reviewed by SUSSMAN (1949) and WIGGLESWORTH (1950). In our experiment, the soft, unpigmented elytra of C-type immediately after emergence were used and it was observed that free and bound tyrosine were both contained in equal amounts in the anterior and the posterior parts regardless of melanin pattern. However, many workers have

stated that the pigment pattern is formed by a difference in tyrosinase activity. GRAUBARD (1933) compared the tyrosinase activities in the larvae and pupae of various body color races of Drosophila. SCHUURMAN (1937) assumed by using *Tenebrio molitor* that the formation of pigment might be due to the difference of enzyme actions. HARIZUKA (1947), ARUGA and KAWASE (1951) and OHNISHI (1954) advocated that the variation of melanin pigment controlled by genic action would be attributed to the difference of tyrosinase activity. WADDINGTON (1941) reported in his observation of various mutants of *D. melanogaster*, that in the pigmentation of adult cuticle, there were two processes of browning and blackening, i.e. one for the tanning and the other for the deposition of melanin. Furthermore, on the basis of his findings, he also believed that the pattern was constructed by partially concentrated blackening and that the mutants showed a remarkable difference in blackening pattern. We also have a concept that both processes could coexist in the cuticle of elytra and that its black pattern was concerned with blackening cuticle.

ARUGA, YOSHITAKE and ISHIHARA (1953) investigated the relationship between uric acid, pterins and melanin, and found that the amount of uric acid in the epidermal cells was inversely proportional to the amount of melanin in the cuticle. ITO (1953) suggested the significance of the relationship among uric acid, pterins and phenol oxidase. In the elytra of the lady beetle, we found about twice as much uric acid in the red part as in the black part. Considering that uric acid is produced from xanthine by xanthine oxidase, attention should be drawn to the interaction between xanthine oxidase and tyrosinase before passing on the relation of uric acid to melanin.

In an in vitro experiment, POLONOVSKI, GONNARD and BORIL (1951) showed that isoxanthopterin specifically inhibited the initial oxidation of dopa. From the viewpoint of physiological genetics, KIKKAWA (1953) has advanced an opinion that there is an intimate relationship among riboflavin, pterin and uric acid, and he has assumed that these substances are produced from a certain common precursor.

MATSUDA (1950) detected folic acid in the pupae of silkworm by a bioassay method and also found that a mutant "lemon" contained more folic acid than wild strains. By a similar method, we also detected folic acid in the pupae of lady beetles. According to the findings of LOWRY, BESSEY and CRAWFORD (1949), folic acid is believed to be decomposed by ultraviolet rays as follows: folic acid → 2-amino-4-hydroxy-6-formylpteridine → 2-amino-4-hydroxy-6-carboxylic pteridine → 2-amino-4-hydroxy pteridine. The last compound in the sequence is converted to isoxanthopterin by the catalytic action of xanthine oxidase. HIRATA, NAKANISHI and KIKKAWA (1950) found isoxanthopterin in the epidermis of the silkworm. ARUGA and YOSHITAKE (1954) recognized some pterins obtained by the decomposition of folic acid in similar material. In frog skin, HAMA (1951) found a fluorescent substance very similar to 2-amino-4-hydroxy-6-carboxylic pteridine. As shown in this paper, we have extracted fluorescent substances from the elytra and bodies of lady beetles. By using paper chromatography, some of these substances have been proved to correspond to P-6-COOH, P-6-CH_2OH, P-6-CH_3, and isoxanthopterin. It is interesting that a large quantity of isoxanthopterin was found in the elytra having little black melanin. It has been also shown that the pterin extracted from the elytra of Coccinella sp. inhibited the tyrosinase activity. This pterin may be

considered to be P-6-COOH on the basis of the result of paper chromatography. The inhibitory action of this substance is similar to that described by GONNARD and SVINÁREFF (1952).

Recently, KIKKAWA, OGITA and FUJITO (1954–55) have worked on the metabolic systems of tyrosine and tryptophane in animals and found that there existed a definite relationship between metals and color pigments. In the elytra of the lady beetle, it has been shown that the red colored part contains a remarkable quantity of molybdenum. Xanthine oxidase has been proved to be molybdoflavoprotein by MACKLER, MAHLER and GREEN (1954), and RICHERT and WESTERFELD (1954). From these findings mentioned above, it is conceived that the xanthine oxidase in the red colored part of elytra may have a high degree of activity.

In the review of copper metabolism in the invertebrates, DETHIER (1950) and MALLETTE (1950) referred to the fact that tyrosinase, an enzyme containing copper, was related to the hardening of cuticle and the formation of pigment pattern. DENNELL (1947) showed that the factor affecting the localization of pigment was the oxidation-reduction potential of the system in the integument of Sarcophaga larvae. This result may possibly account for the inhibitory action of the xanthine oxidase system on tyrosinase at a low rH value.

In view of the analytical data on the metals contained in elytra, it seems to be that the distribution of copper may be uniform in the process of hardening and pigmentation. At this point, it may be speculated that tyrosinase is partially inhibited by an increased activity of molybdoflavoprotein, which may be originally controlled by genic action. Figure 8 summarizes the above discussion and the possible mechanism of pigment pattern formation in elytra.

Our experiments using nitrogen mustard indicate that it is at the late egg stage that genic action controlling pigment pattern is manifested. According to GEIGY's work reviewed by WIGGLESWORTH (1950), the epidermal structure of the adult blowfly appears to be determined in the early developmental stage, e.g. egg stage.

The idea that genotype controls the specificity of protein is the general concept of the physiology of gene action. A similar one may be affirmed in the formation mechanism of pigment pattern.

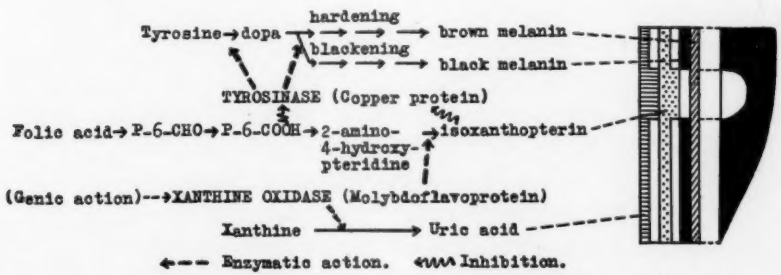

FIGURE 8.—The diagram illustrating the mechanism of pigment pattern formation in the elytra of lady beetle.

SUMMARY

1. The mechanism of the formation of elytral patterns in lady beetles, *Harmonia axyridis* Pallas and *Coccinella bruckii* Mulsant, was studied by means of genetical and biochemical methods. Several variant types of elytra (C-type, S-type, A-type and s-type) of *Harmonia axyridis* were presented as multiple alleles; the genic action controlling these characters was assumed.

2. Tyrosine, the substrate of melanin, was extracted from the soft, unpigmented elytra of C-type immediately after emergence, isolated by use of action ion-exchange resin and measured quantitatively. The amount of free tyrosine was increased before the hardening and darkening of the cuticle and its distribution over the elytra appeared to be independent of the formation of melanin pattern.

3. Uric acid was extracted from the hard, pigmented elytra and from the bodies. It was measured after being treated in the same manner as tyrosine. The content of uric acid was higher in the s-type elytra than in the C-type elytra. However, the melanin contents of both types were reversely proportional to those of uric acid. Same quantities of uric acid were extracted from the bodies of these two types.

4. The fluorescent substances were extracted and separated by paper chromatography. The photo-decomposed substances of folic acid such as the pteridines, e.g. P-6-COOH, P-6-CH_2OH, P-6-CH_3, and isoxanthopterin were detected. The fluorescent substance extracted from the soft, unpigmented elytra of Coccinella sp. with acetone had an inhibitory effect on tyrosinase and the behavior of its inhibition coincided with that of 2-amino-4-hydroxy-6-carboxylic pteridine. The isoxanthopterin was extracted from the elytra of s-type and Coccinella sp.; this substance was extracted from the elytra of C-type in lesser amounts.

5. The elytra of C-type were horizontally divided into two parts along the lower margin of the red colored pattern and equal amounts of each part were burnt to ashes by wet oxidation. The quantities of metals, e.g. molybdenum, copper and cobalt, were measured. The content of molybdenum was most remarkable in the red colored region, but the quantity of copper in each part was almost equal.

6. The eggs laid by the F_1 hybrids between the homozygous S-type and s-type lady beetles were smeared with 0.05 and 0.1 percent of nitrogen mustard. A few individuals identical with respect to having C-type elytra developed from these treated eggs. It was speculated that determination of the elytral pattern might be initiated in the egg about 48 hours after oviposition.

7. The interaction between the metabolic systems related to the process of pigmentation was discussed, and the mechanism of pattern formation was presented diagrammatically. The genic action yielding the pattern was assumed to be closely related to the determining factor in the localization of xanthine oxidase.

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SPONTANEOUS MUTATIONS AFFECTING THE KILLER CHARACTER IN PARAMECIUM AURELIA, VARIETY 4[1]

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KILLER paramecia release into the medium in which they live a poison, paramecin, to which they are resistant but which kills other *sensitive* paramecia (SONNEBORN 1939, 1943). Two spontaneous mutations of the killer character are analyzed in this paper. One of these proves to be a mutation of kappa, the cytoplasmic genetic particle determining the killer trait (SONNEBORN 1946); the other is a nuclear mutation, the first such mutation of the killer trait to be found.

Kappa mutations have been reported by several investigators. They can be placed in four categories: 1) changes in killing ability (PREER 1948; DIPPELL 1950; HANSON 1954), 2) changes in maximal reproductive rate (DIPPELL 1950), 3) changes in antigenic properties (HANSON 1954), 4) changes in resistance to aureomycin (EHRET and POWERS 1952). The kappa mutant analyzed here falls into the second category.

As long known (SONNEBORN 1943) the maintenance of kappa in variety 4 depends on the presence of a nuclear gene, *K*. In homozygotes for *k*, kappa is sooner or later irreversibly lost. Stocks collected in nature have been found to differ with respect to the presence of *K* or *k*, but no spontaneous or induced laboratory mutations at this locus have been reported. The nuclear mutant analyzed in this paper involves a spontaneous change at or near this locus in material originally homozygous for *K*.

MATERIALS AND METHODS

Stock 31 of variety 8 (LEVINE 1954) was used as the standard sensitive to detect whether tested cultures were killers. In like manner, stock 51 of variety 4 was used as the standard killer (SONNEBORN 1943) to detect whether tested cultures were sensitives. Stock 51 is a hump killer (DIPPELL 1950). The methods of making these tests, like the methods of cultivation, crossing, controlling fission rates, inducing autogamy, removing kappa, and obtaining specific antisera, are fully described by SONNEBORN (1950).

For genetic analysis, stock 51 was used as the standard *KK* culture and the derived stock d186 as the standard *kk* culture. This latter carries the *k* gene of stock

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29 in a genetic background approximately isogenic with that of stock 51 (DIPPELL 1950).

The first mutant is referred to as line 468. It arose as the 468th autogamous isolate in the F_2, following a cross between two homozygous *KK* killers, stocks 47 and 51. This work was carried out in 1946 by SONNEBORN and DIPPELL who set aside clone 468 for later study because it unexpectedly proved to be a sensitive, unlike all the other F_2 cultures, which were, as expected, killers. Line 468 was finally studied by the present author four years later. In the meantime, of course, it had undergone many self-fertilizations and perhaps conjugations among its own descendants.

The second mutant arising in stock 51, was turned over to the author by SONNEBORN and SCHNELLER after noticing that it deviated from the normal in two respects: 1) it was a weaker killer although of the same hump type, 2) it gave rise to sensitive progeny when grown rapidly at 27°C, while normal killers did not. In accordance with DIPPELL's practice of designating kappa mutants by the name of the stock of origin followed by the symbol m (for mutant) and a number, this mutant is designated 51m7. Mutants 51m1 to 51m5 are dealt with by DIPPELL (1950) and 51m6 by HANSON (1954).

Of the cytological methods employed, DIPPELL and CHAO's modification of DeLamater's basic fuchsin technique is found in SONNEBORN's 1950 paper. Study of the agglutination of kappa particles by specific antiserum was carried out by techniques reported by PREER and STARK (1953) and HANSON (1954).

A SPONTANEOUS NUCLEAR MUTATION AFFECTING THE KILLER CHARACTER

The single sensitive line 468 appeared in the autogamous F_2 after crossing two homozygous *KK* killers (stock 47 × stock 51). Two explanations of this surprising result seemed *a priori* possible. 1) For reasons unknown, kappa might have been lost without any relevant nuclear mutation. 2) Kappa might have been lost as a consequence of a nuclear mutation of *K* to *k*. In the latter case the mutation would have been a recessive present in heterozygous form in the animal that gave rise at autogamy to line 468. Other conceivable types of nuclear mutation will be discussed later. The explanation based on kappa loss would perhaps seem the more likely, since it is known that kappa can be lost under certain conditions without gene change, and since neither spontaneous nor induced nuclear mutations affecting the maintenance of kappa have ever been found. Yet the association of the sensitive with the occurrence of autogamy, which brings about homozygosity, raised the suspicion of a nuclear mutation and this possibility was examined first.

If line 468 differs from the ancestral killers (stocks 47 and 51) by a single mutant gene, backcrossing 468 to either ancestral stock should yield a 1:1 ratio of killers to sensitives in the next autogamous generation. This was tested, using the backcross to stock 51. In agreement with the hypothesis, killers and sensitives segregated in the autogamous F_2; but there was a significant departure from the theoretical 1:1 ratio, killers appearing in excess. It now appears, in view of the subsequent work of CHAO (1954), that this might have been due to the prolonged maintenance of kappa by some of the genetically sensitive segregants. On the other

hand, there might have been greater viability of the killer segregants. As pointed out by SONNEBORN and DIPPELL (1946), the F_2 generation following crosses of natural stocks always yields a considerable proportion of non-viable clones and the proportion of non-viability is progressively decreased by approaching isogenicity through repeated backcrosses. The F_2 in which line 468 arose showed 55% mortality and there was still considerable, though less, mortality at the autogamy following the first backcross. To avoid complications possibly due to differential viability of the segregating classes, further backcrosses—each followed by autogamy with selection of a sensitive segregant for the next backcross—were carried out. After the third backcross, the mortality at the following autogamy was less than 10%, only 95/1025 clones dying. Among the survivors were 485 killers and 445 sensitives. This does not differ significantly from a 1:1 ratio; chi square is 1.324 and P lies between .2 and .3. The expectation, on the hypothesis that line 468 differs from its ancestors by a single mutant gene unable to support kappa, was thus fulfilled. That the sensitivity of 468 and its sensitive progeny was not characterized by low concentration of kappa, but by total absence of kappa, was confirmed by staining which failed to reveal the presence of kappa in these sensitives.

For the further investigation of this hypothesis, one of the sensitive segregants was used instead of the original line 468. The essential factor for determining sensitivity of this new line was of course derived from line 468, but many other irrelevant stock 47 genes carried by the original line 468 were replaced by stock 51 genes as a result of the three backcrosses and autogamies. The advantage of such material for work involving crosses to stock 51 and its isogenic derivatives has already been pointed out. We shall refer to this derivative of 468, and all progeny that carry the same factor for sensitivity, by the same symbol, i.e. 468.

Thus far the analysis gives no clue as to whether the mutation in 468 is at the locus of K, or elsewhere. If it is elsewhere, then 468 still carries K and its sensitivity is due to a mutation at a previously unknown killer locus which may be symbolized provisionally as A. On this hypothesis 468 is homozygous for the recessive, aa, and the ancestral stocks are homozygous for the dominant, AA. On the other hand, if the mutation is at the K locus, then the mutation of 468 would be from K to an allele that can be tentatively designated as k. These alternatives were tested by introducing the stock d186 into the analysis. This stock is sensitive because it is homozygous for gene k. The test employed here is explained in figure 1. It starts by crossing sensitive 468 to stock 51 (KK, killer) and selecting a heterozygous F_1 killer to test for genotype. On one hypothesis this heterozygote is Kk; on the other it is $KKAa$. These alternatives can be distinguished by crossing it to the standard d186, which is $kkAA$. From such a cross no genotypes determining loss of kappa and the killer trait can be directly obtained if 468 is $KKaa$; but, if 468 is kk, half the exconjugant pairs should be kk and the original killer members of these pairs should lose kappa and become sensitives. The latter was the result obtained: the killer member of 39 of the 73 pairs became sensitive. As a control, d186 was crossed to stock 51 and a heterozygous F_1 killer was backcrossed to d186. As expected, about half (25 out of 58) of the original killer members of the backcross became

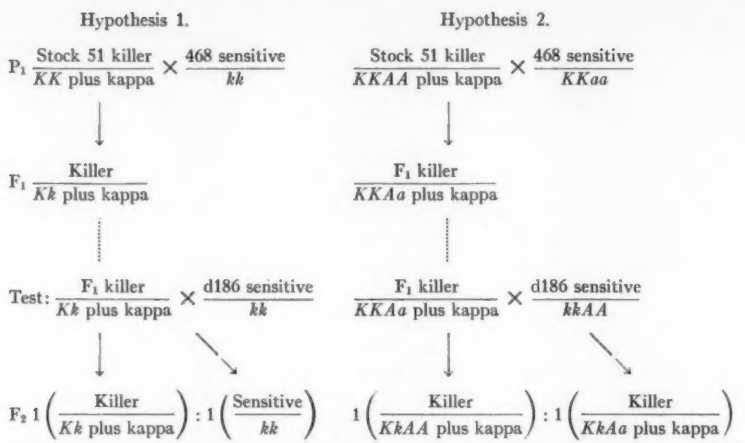
Hypothesis 1.
Hypothesis 2.
P1 Stock 51 killer / KK plus kappa × 468 sensitive / kk
Stock 51 killer / KKAA plus kappa × 468 sensitive / KKaa
F1 Killer / Kk plus kappa
F1 killer / KKAa plus kappa
Test: F1 killer / Kk plus kappa × d186 sensitive / kk
F1 killer / KKAa plus kappa × d186 sensitive / kkAA
F2 1 (Killer / Kk plus kappa) : 1 (Sensitive / kk)
1 (Killer / KkAA plus kappa) : 1 (Killer / KkAa plus kappa)

FIGURE 1.—Method of testing the genotype of mutant line 468. Hypothesis 1: Sensitive 468 differs from stock 51 killers by mutation of K to k. F_2 progeny of killer member of test cross are either killer or sensitive. Hypothesis 2: Sensitive 468 differs from stock 51 killers by mutation at a previously unknown killer locus A. On this hypothesis stocks 51 and d186 (known to differ from 51 at a single locus defined as K) must both be AA. F_2 progeny of killer member of test cross are all killer.

sensitives, showing that d186—as already known—differs from 51 at a single locus, the K locus by definition. Hence the mutation of 468, if it involves only a single locus, must be at the same locus.

The possibility of interpretations more complex than a single gene mutation in 468 remains to be considered. That 468 does not carry two or more unlinked or loosely linked sensitive mutations is already clear from the 1:1 ratio at autogamy after crossing 468 to stock 51 (see above). With more than one mutation not closely linked, there should have been an excess of sensitives at that autogamy. On the other hand, if two or more closely linked mutations were involved, the 1:1 ratio would be approached more and more closely the closer the loci. The coincidence of two mutations in 468 is of course *a priori* so unlikely as to be hardly worth considering unless they were correlated with a small inversion.

Thus far only gene mutations have been considered. But so far as now known, k is simply an inactive allele of K, and a loss of K obviously might be indistinguishable from a mutation to k. This is a possibility in view of DIPPELL's (1955) findings of unequal chromosome numbers in different stocks of variety 4. These differences could cause aberrancies in the meiotic process at autogamy, resulting in loss or gain of chromosomal material, and this could be the basis for the regular occurrence of non-viability in crosses between diverse natural stocks. The mutant 468 arose in the F_2 of a cross between two stocks known to differ in chromosome number and there was non-viability in the F_2 and the subsequent backcrosses. Hence losses and gains of chromosomal material might be expected. Furthermore, the excess of killers in the autogamies following the first few backcrosses could have been due to the

poorer chance of survival of the sensitives due either to their lack of a gene, or several genes, or even a whole chromosome, or, less likely perhaps, to their gain of some such material. Studies have not been made to distinguish between absence or gain of genetic material and a gene mutation.

MUTATION OF KAPPA

The mutant killer 51m7 was found to show its characteristic features only under certain conditions of culture. When grown at 27°C with limited food, enough to permit only one fission per day, it is not only a typical hump killer, but as strong a killer as normal killers. On the other hand, after growth at 27°C for ten days with excess food, permitting maximal growth rate at this temperature little or no killing activity remains and some of the animals become irreversible sensitives. The phenotype of 51m7 can, however, be most readily characterized by rapid growth at 31°C. The rapid growth is achieved by daily subculture of the animals into fresh culture fluid. Starting with the animals from a tube culture that is being maintained at one fission per day at 27°C, one drop—about 0.05 ml containing about 30–50 animals—is transferred to roughly 1 ml of fresh culture fluid. This permits another 4 to 5 fissions before the food is exhausted. These 4 to 5 fissions are usually completed within 24 hours, at the end of which time one drop from this culture is transferred again to one ml of fresh culture fluid. After another day's growth a third similar subculture is made. This third day's subculture is left undisturbed at 31°C for a fourth day and is removed to 27°C on the fifth day to test it for the killing ability of the animals contained in it. A culture of the mutant killer treated in this manner can be either a weak killer culture, a resistant culture, or a sensitive one. Normal 51 killers undergoing this same treatment all remain strong killers with perhaps a slight reduction in killing ability.

These observations can be understood if it is postulated that the mutation in 51m7, whether nuclear or cytoplasmic, affects the reproductive rate of kappa in such a manner that kappa cannot multiply as rapidly as the animals under conditions of high fission rates. The kappa is reduced or lost by dilution. This interpretation is supported by the fact that staining 51m7 animals after this period of rapid growth reveals a great reduction in the kappa particle concentration in each animal. Whereas homozygous 51m7 animals grown at 27°C at one fission per day possess on the average 400–500 kappa particles per animal, after the period of rapid growth at 31°C they contain a maximum of 100 particles each. PREER (1946) and SONNEBORN (1946) have shown that quantitative variation in the amount of kappa can result in correlated variations in the amount of killing.

The question of whether a cytoplasmic or nuclear mutation had occurred in the origin of 51m7 was first attacked by testing for a nuclear mutation in the way done earlier by DIPPELL (1950). To do this, it was necessary to avoid the introduction of any other kappa into the analysis and yet to work with a genotype known to be capable of maintaining normal killers. The necessary material was obtained by removing kappa from the standard stock 51 killer, using the technique of exposure to 33.8°C (SONNEBORN 1950). The resulting culture, a *KK* sensitive of mating type VII and serotype D, was crossed to the weak killer 51m7 of mating type VIII and

a serotype other than D, in order to obtain an F_1 heterozygous for any possible nuclear mutation carried by 51m7. This F_1 was then allowed to produce an F_2 by autogamy and the F_2 clones were subjected to the technique of rapid growth at 31°C (see above) to ascertain whether the cultures were strong killers of the normal sort or weak killers of the 51m7 type. These two types of killers should segregate in the F_2 if 51m7 carried a nuclear mutation.

The results are set forth in table 1, along with data on controls. The controls consisted of an F_2 derived from the cross of the same *KK* sensitive clone to a standard killer (*KK*) of stock 51, mating type VII and non-D serotype. As three F_1 pairs were used in each cross and as the killer member of each F_1 pair was used to provide

TABLE 1

Results of rapid growth at 31°C on the killing ability of F_2 animals derived by autogamy from the F_1 from crosses of the weak killer and normal 51 killer to KK *sensitives*

Cross	F_2 before rapid growth at 31°C			
	strong*	intermediate	weak	died
Mutant killer × *KK* sensitive				
1	24	0	0	0
2	24	0	0	0
3	24	0	0	0
Totals	72	0	0	0
51 killer × *KK* sensitive				
1	23	0	0	1
2	24	0	0	0
3	24	0	0	0
Totals	71	0	0	1
Cross	F_2 after rapid growth at 31°C			
	strong	intermediate	weak	died
Mutant killer × *KK* sensitive				
1	0	0	24	0
2	0	1	23	0
3	0	0	24	0
Totals	0	1	71	0
51 killer × *KK* sensitive				
1	19	4	0	1
2	24	0	0	0
3	22	2	0	0
Totals	65	6	0	1

* Strong = more than 100 sensitives killed in 48 hours, intermediate = 50 to 100 sensitives killed in 48 hours, and weak = less than 50 sensitives killed in 48 hours.

24 autogamous F_2 clones, there were 72 experimentals and 72 controls altogether. Table 1 shows that all of these clones (except one that died) were strong killers before the period of rapid growth at 31°C and that after such growth, none of the experimentals was a strong killer while none of the controls was a weak killer. The one intermediate experimental culture and two of the six intermediate controls were retested and found to fall into the expected classes: the experimental culture was a weak killer, the two controls were strong killers. (The criteria for strong, weak, and intermediate killers are noted in table 1.) There is thus no segregation of two types of killers in either kind of F_2: the experimentals were all weak killers like 51m7 and the controls were all normal strong killers. Consequently, the data offer no support to the hypothesis that the mutation in 51m7 was nuclear.

Failure of segregation could conceivably have resulted from either of two causes, both of which can be excluded or rendered unlikely. First, the conjugants used as the F_1 might have involved matings between two killers rather than between killer and sensitive, for selfing pairs do occur occasionally. This was excluded by testing the serotypes of the two members of each exconjugant pair. In every pair one member was serotype D and the other was not, in agreement with the serotypes of the parental killer and sensitive cultures, respectively. Second, even though each pair consisted of a killer and sensitive, each mate might have fertilized itself (cytogamy, WICHTERMAN 1953) instead of undergoing cross-fertilization. This was rendered very unlikely by the results of killer tests made on the two clones produced by each F_1 pair of conjugants. In all six pairs, *both* members proved to be killers. This happens only when cytoplasm is exchanged between mates and self-fertilization is not known to occur when the mates are so intimately united as to exchange cytoplasm. There thus seems to be no reasonable explanation for the failure of segregation in the F_2 except that 51m7 is not a nuclear mutant.

With the elimination of the hypothesis of a nuclear mutation, a cytoplasmic mutation is indicated. Two possibilities must be distinguished here: a mutation of kappa itself and a mutation of some other part of the cytoplasm. Since kappa is known to be mutable, the possibility of a new kappa mutant seemed likely and was tested first. To carry out such a test, it was necessary to construct a type of animal which contained kappa from 51m7 and kappa from another kind of animal not showing the 51m7 character. Then tests could be carried out to discover whether two types of kappa were present and maintained their diverse characters in a common cytoplasm. Since the kappa of 51m7 is distinguished from normal kappa by its lower maximal reproductive rate, the problem resolves itself into discovering whether the constructed type of animal contains two kinds of kappa differing in this way. The test was actually made sharper by using as the fast kappa one which was distinguishable in another way, namely, by its failure to confer either killing or resistance on its possessor. This is the kappa mutant known as pi (HANSON 1954).

The test material was constructed by crossing 51m7 to sensitive animals bearing pi and selecting conjugant pairs that had exchanged cytoplasm (SONNEBORN 1950). If the kappa of 51m7 is a "slow" mutant, then the exconjugants from this cross should have two kinds of kappa: "slow" killer kappa and "fast" non-killer kappa (or pi). The test consists of subjecting these animals to the technique of

rapid growth at 31°C. If only the killer kappa is lost, as it is in 51m7 animals under these conditions, then the animals would cease to be killers but would retain the non-killer kappa. This would prove that there were in fact two kinds of kappa present in a common cytoplasm, one slow-growing and one fast-growing, the former controlling the killing and the latter not. As controls, the same conditions of culture were used for 51m7 and for the pi mutant that provided the fast, non-killer kappa.

Observations made at various times during the rapid growth at 31°C showed parallel progressive decrease in killing capacity of 51m7 and of the experimental culture bearing kappa from two sources. Eventually all lines of both of these cultures became sensitive. At this time the experimental animals still contained more than 200 particles of kappa per animal, while there were less than ten particles of kappa per animal in 51m7. Clearly the kappa still present in large amounts in the experimental lines was nearly all pi, for such quantities of 51m7 kappa would have made the animals killers, not sensitives. The loss of kappa during rapid growth at 31°C in 51m7 animals is thus a specific character of the kappa in these organisms, a character which is distinctively maintained even when this kappa is present in a cytoplasm containing kappa of another type. In other words, the mutant 51m7 appears to be so by reason of a mutation in kappa itself. This conclusion can be avoided only by making more complex and less probable assumptions about other sorts of cytoplasmic mutations. At present this seems inadvisable since the available data are satisfactorily explained by the simpler hypothesis of a kappa mutation differing only in its upper limit of reproductive rate.

In all other respects thus far examined, the kappa in 51m7 is exactly like normal kappa of stock 51. (1) Both produce the same kind of hump killing. (2) Both are lost at 35°C without rapid growth. (3) Both have the same morphology in vivo (bright phase) and in whole mounts prepared with basic fuchsin. (4) Both are agglutinated by antiserum prepared against the kappa of stock 51.

DISCUSSION

Analysis of the mutant line 468 led to the conclusion that a mutation had occurred from *K* to *k* or that the *K* locus was lost. The latter alternative was considered in view of DIPPELL's (1953) work on the chromosomal differences between stocks of *P. aurelia* and their implications for inter-stock hybrids and their sexual progeny. Here it should be pointed out that similar considerations apply to the stock d186 used above as the standard *kk* stock. The *k* gene of this stock was derived from the wild stock 29 and introduced into the genome of wild stock 51 in preparing the derived stock d186. The origin of stock 29 is of course unknown; its *k* gene could be an allele of *K* or a loss of *K*. Further, even if a true allele (*k*) had been present in stock 29, it would be subject to the same hazards of loss in the process of introducing it into the genome of another natural stock (51) as those considered in connection with the mutant line 468. Thus, there is at present no clear evidence whether what is called *k* in stock 29, in d186, or in line 468 is an allele of *K* or mere absence of *K*. Moreover, the possibility of getting a decision by

a search for reverse mutations seems completely impracticable because there is no selective method available for spotting mutations in this direction.

The mutant 51m7 presents a different problem. Its ancestor was a normal killer of stock 51 and therefore contained hundreds of kappa particles. How then are we to understand the origin of mutant 51m7, which also contains hundreds of kappa particles, but of a different type? Presumably the mutation arose initially in a single kappa particle. How then did the descendants of the mutant particle overgrow and eventually displace all the unmutated kappa? The problem seems superficially all the more perplexing since the only detected difference between the two kinds of kappa is in the *slower* growth of the mutant under certain conditions. The same problem was encountered by DIPPELL (1950) in her study of kappa mutants. The present author has made a study of this problem. The results will be published elsewhere.

SUMMARY

Two new mutations affecting the killer character in *Paramecium aurelia*, variety 4, have been analyzed. One is a spontaneous nuclear mutation involving loss of kappa, whose exact nature involves one of the following alternatives: a) gene mutation at or closely linked to the *K* locus such that animals homozygous for the mutant gene can no longer support kappa, or b) loss of the *K* locus. The other mutation is localized in kappa itself and is characterized by a reduced maximal reproductive rate of the kappa particles.

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SEGREGATION IN NEW ALLOPOLYPLOIDS OF GOSSYPIUM. I. THE R_1 LOCUS IN CERTAIN NEW WORLD-WILD AMERICAN HEXAPLOIDS

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ALLOPOLYPLOIDS and their derivatives have attracted much attention during the last three decades because of their importance in the evolution of wild and cultivated plants and as tools in crop breeding. It is quite surprising that the genetic behavior of newly synthesized allopolyploids has rarely been investigated. Best known are those examples which show little segregation and are more or less true breeding like *Nicotiana digluta* (CLAUSEN and GOODSPEED 1925) while the opposite extreme is exemplified by amphidiploid *Fragaria bracteata* × *vesca* with random segregation for an anthocyanin factor *P* (YARNELL 1931; however CLAUSEN, KECK and HIESEY, 1945, regard this as a case of autotetraploidy). Such clear-cut behavior has not always been encountered; e.g., the classical example of *Primula kewensis* produced an intermediate amount of segregation which was ascribed by NEWTON and PELLEW (1929) to the formation of some quadrivalent chromosome figures in meiosis. An interesting situation was reported by SKALIŃSKA (1935) in amphidiploid *Aquilegia chrysantha* × *flabellata* where certain parental characters segregated while others did not. Similar behavior occurred in a tetraploid hybrid between *Rubus rusticanus* and *R. thyrsiger* (CRANE and DARLINGTON 1927). Several cases are known where segregation for taxonomic characteristics or for fertility has been observed or where dwarfs occurred in the progeny of raw allopolyploids, but as the genetic basis was not determined no precise factorial analysis was possible (BUXTON and DARLINGTON 1931; CUA 1952; JACOB 1952; STEBBINS and VAARAMA 1953; and others). FAGERLIND (1937) has attempted to correlate quantitatively cytological behavior with genetic segregation in 4n *Galium Mollugo* × *verum*, but as the segregating population consisted of only 57 plants his considerations were largely theoretical.

The genus Gossypium is particularly well suited for such studies. A fair number of qualitatively inherited characters have been thoroughly investigated in the past and allopolyploids can be synthesized with relative ease from species of various degrees of taxonomic affinity. The results should not only bear on some theoretical questions, discussed below, but also reveal to the cotton breeder the ease, or the difficulty, with which genes from wild species can be incorporated in cultigens.

A number of raw allopolyploid combinations are now under study. Previously RHYNE (1951) had obtained evidence for some segregation in certain of these com-

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binations. The present efforts are directed towards obtaining more precise information as to the extent and the nature of this segregation. This work has progressed furthest with several hexaploids which were duplex (e.g. *RRrr*) for R_1 alleles which control formation of anthocyanin in certain parts of the plant. The genetics of these hexaploids is the subject of the present paper.

MATERIALS AND METHODS

Four species were used, two of them wild and the other two cultivated:

G. thurberi Tod. is widely scattered over southeastern Arizona and certain parts of northern Mexico. Its vegetative parts are green and it carries a small, variable red spot at the base of the petal. This spot does not appear in hybrids with *G. hirsutum*.

G. raimondii Ulb. comes from the western slope of the Peruvian Andes. The vegetative parts form very little anthocyanin, but at the bottom of the petals there is a very large deep carmine spot; filaments and the base of the androecium are similarly colored. The absence of plant body pigment is recessive while the flower pigmentation is dominant in all interspecific combinations. These two species are diploids (2n = 26) and carry the D genome of chromosomes (BEASLEY 1942). They were selected from the eight known D species because they represent among the wild American Gossypiums the extremes in both geographical origin and morphology. The others were also not suited because they gave with *G. hirsutum* either lethal hybrids like *G. hirsutum* × *davidsonii*, or the polyploid combinations rarely matured seeds like various 6n *G. hirsutum-armourianum* combinations.

The next two species are the New World cottons which produce the main crop of cotton lint and seed oil in the world today; they are natural allotetraploids (n = 26). Their genomes have been designated AD by BEASLEY (1942).

Various genotypes of *G. hirsutum* were employed: S4025 is a combination of several dominant marker genes of which only the anthocyanin factors are pertinent here. Of these, R_1 produces a red pigment in the plant body and on the margin of the petals, but no petal spot. This gene is located in the D genome. S4025 also has R_2, an A genome factor for small petal spot. Strain S5082 is a combination of various recessive markers without anthocyanin in either leaf blades or petals; i.e., it carries r_1 and r_2. Linked with r_1 it also has *cl*, the gene for clustered flowers. (S5082 could be regarded as not quite pure *hirsutum*; the factor for crinkled leaf carried by this stock came originally from *G. barbadense*, but many backcrosses to *G. hirsutum* followed and crinkle is also independent of the r_1-cl_1 linkage which alone is of interest here.) The third marker strain used, SM4, also is a combination of recessives, of which the linkage of r_1 and yg_1 (yellow green: RHYNE 1955) will be mentioned later. SM2, like S5082 and SM4, possesses r_1. This line also carries several dominant markers and was synthesized from *G. hirsutum* sources quite different from those of the other lines. In addition to these four marker strains which were incorporated into synthetic hexaploids certain commercial strains carrying r_1 and r_2 were used as test plants (Coker, Empire, Stoneville).

G. barbadense L. was represented by a single strain, TZVR, of Sea Island cotton with green plant body (r_1) and a small spot at the petal base produced by R_2 in the A genome.

Hexaploids were produced from triploid hybrids between the cultivated and the wild species by the application of an aqueous mixture of colchicine (.5%), tragacanth (4%), and a small amount of thymol to the growing points of young seedlings as recommended by SCHWANITZ (1949). Best results were obtained when the treatment was repeated after 24 hours. A large proportion of the treated hybrids produced hexaploid tissues. Successfully doubled plants were transplanted to beds in the greenhouse or to large 10-gallon containers where there was no extreme limitation of root growth. In some cases the hexaploids were multiplied by grafting on Upland stock.

G. raimondii blooms only during short days as do the hybrids with New World cottons and therefore must be grown indoors under our conditions. All testcrosses were made in the greenhouse from November until May for a period of several years. *G. thurberi* derivatives were subjected to the same regime. Because of low seed production and of space restrictions it was impossible to limit crossing to a short period with uniform conditions and yet get sufficiently large progenies, as might have been desirable.

In the tables concerned with segregation data only those plants are listed which reached the blooming stage. Only a few plants failed to flower and this was more likely due to an environmental handicap than to a genetical one as New World-Wild American hybrid derivatives tend to bloom only towards the end of the season. A few others died at various stages before, or sometimes after, transplanting. Among these were a few rare monstrosities with greatly swollen cotyledons and no active growing point which probably were the result of unbalanced chromosome complements.

RESULTS

Fertility of the hexaploids

In any study of genetical segregation it is important to see whether any appreciable abortion occurs in the material and then it is necessary to ascertain to what extent the ratio may be biased by differential survival. This elementary precaution has been overlooked in most studies of segregation in allopolyploids. Table 1 gives

TABLE 1

Seed fertility in hexaploid × G. hirsutum *crosses**

Hexaploid	No. of plants	Aborted bolls	Retained bolls	Germinable seeds per boll	
				Mean number†	Range
G. hirsutum S4025-*raimondii*	2	not counted	51	8.0	0–19
S5082-*raimondii*	6	603	26	4.7	0–16
SM4-*raimondii*	1	66	19	4.8	1–14
SM2-*raimondii*	4	175	55	9.1	0–23
G. barbadense TZVR-*raimondii*	3	26	33	6.0	0–11
G. hirsutum S4025-*thurberi*	2	not counted	27	7.9	0–16

* All hexaploids were grown in bed in greenhouse; data are from crosses made during one entire winter season.

† Bolls with 0 seeds were not included in mean as they were mostly shed at an early stage.

an idea of the amount of sterility encountered in the Gossypium hexaploids. In *G. hirsutum-raimondii* combinations the large majority of bolls fell off at varying intervals between pollination and maturity. In *G. barbadense-raimondii* hexaploids a little more than half of the pollinated bolls stayed on which also seemed to be the case with 6n *G. hirsutum-thurberi* though no counts were made. The loss of bolls must be ascribed, at least in part, to physiological abscission which is common even in pure species of Gossypium and is much more pronounced during certain times than during others in both species and hybrids.

Genetically more significant could be the fact that only a few seeds in each boll reached maturity whereas the majority aborted at various stages during development. Thus a maximum of 13 germinable seeds were obtained from 6n S5082-*raimondii* which holds 20 to 32 ovules per boll, but the average set was much lower, namely 4.7. Similarly, at most 16 good seeds were in bolls of 6n S4025-*thurberi*, or an average of 7.9, whereas boll capacity was found to range from 17 to 28 ovules. Ovule counts were not made from the remaining hexaploids, but many ovules aborted in all of them. The frequency of bolls with a large number of germinable seeds was rather high; in a few almost every ovule grew to maturity (table 2). The number of seeds per boll did not fit any random distribution as it would were the chance for an embryo to reach maturity independent of others and dependent on its genotype alone. Presumably, physiological factors were also operative in determining the number of seeds in a boll and one might adduce the recent observation by WEAVER (1955) of interactions between seeds in a boll in which developing seeds stimulated neighboring ones.

Male sterility was also high. It has been quite impossible to obtain any coherent data as the amount of pollen abortion varied extremely from flower to flower on the same plant, even from anther to anther in the same blossom. Anthers without any pollen of normal appearance occurred most frequently in the *G. hirsutum-thurberi* hexaploids and there pollen samples with more than one third good pollen have rarely been obtained. Anthers with more than 50% normal pollen grains were frequent in the *G. hirsutum-raimondii* hexaploids. This pollen failed, however, to produce germinable seeds when applied to flowers of *G. hirsutum*; successful crosses were made by using hexaploids as females. From such crosses the mean number of

TABLE 2

Frequency distribution of germinable seeds per boll from the 6n G. hirsutum-raimondii *crosses in table 1**

Seeds/boll	Bolls	Seeds/boll	Bolls	Seeds/boll	Bolls	Seeds/boll	Bolls
1	15	7	9	13	5	19	2
2	16	8	11	14	4	20	1
3	13	9	7	15	4	21	2
4	14	10	2	16	4	22	0
5	11	11	10	17	1	23	2
6	10	12	5	18	3	Total	151

* Tests of heterogeneity between hexaploid combinations and between plants in hexaploid combinations were non-significant at the 5% level.

TABLE 3

Seed fertility in 6n × 6n crosses

Crosses	No. bolls	Germinable seeds per boll	
		Mean no.	Range
6n *G. hirsutum-raimondii* × 6n *G. hirsutum-raimondii*	14	6.4	1–20
6n *G. hirsutum-thurberi* × 6n *G. hirsutum-raimondii*	63	8.6	2–22

TABLE 4

Segregation for petal spot in 6n New World-raimondii × G. hirsutum

Female parent		Number plants		1163:134 chi square
Combination	Plant no.	*RAI* spot	No *RAI* spot	
6n *G. hirsutum* S4025-*raimondii*	S5094-1	168	22	0.33
	S5094-2	194	20	0.22
	5 plants†	136	16	0.01
6n *G. hirsutum* S5082-*raimondii*	1-209-A	113	21	4.13*
	3 plants†	47	3	1.01
6n *G. hirsutum* SM4-*raimondii*	2 plants†	28	6	1.97
6n *G. hirsutum* SM2-*raimondii*	3-116-B	47	4	0.34
	3-116-C	141	18	0.17
	3-116-D	189	15	1.95
6n *G. barbadense* TZVR-*raimondii*	3-111-A	71	6	0.53
	3-111-C	29	3	0.03
Totals		1163	134	10.69

* Significant ($P = .05 - .01$) chi square values are designated by * and highly significant ($P < .01$) values by ** throughout this paper.

† Data from several small families combined.

seeds per boll and the range were of the same magnitude (table 3) as in the crosses described above.

6n G. hirsutum-raimondii

Hexaploids 6n *G. hirsutum-raimondii* were of the genetic constitution $R_1R_1R^{RAI}R^{RAI}$ or $r_1r_1R^{RAI}R^{RAI}$, depending on whether the *hirsutum* parent carried R_1 or r_1. The *G. raimondii* allele R^{RAI} controls the production of a large red spot at the base of the petals and of red pigment in the filaments ("*RAI* spot"). Offspring from backcrosses of these hexaploids to recessive (r_1r_1) *hirsutum* may have the *RAI* pigmentation or they may not. Classification of 1297 plants from crosses in which hexaploids were used as females is given in table 4. Of these, 1163 carried the spot and 134 did not; this result differed very significantly from a 5:1 ratio expected with random chromosome segregation (chi square = 37.5; df. = 1). All but one of the families could be fitted to the observed total of 1163:134 and were homogeneous. Thus, but for one exception, the spotless plants were uniformly fewer than would be expected with randomness regardless of the genetic line, or even species, of New World cotton used in the hexaploid.

TABLE 5

Segregation in testcrosses of 6n G. hirsutum *S4025*-raimondii
($R_1R_1R^{RAI}R^{RAI} \times r_1r_1$)

Plant no.	Trisomic offspring			Exceptional offspring			
	$R_1R_1r_1$* (1)‡	$R_1R^{RAI}r_1$ (2)	$\frac{R^{RAI}}{R^{RAI}r_1}$* (3)	R_1r_1 (4)‡	$R^{RAI}r_1$ (5)	$R_1R_1R^{RAI}r_1$ (6)	$\frac{R_1R^{RAI}}{R^{RAI}r_1}$ (7)
S5094-1	21	161	5	1	2		
S5094-2	17	176	16	3	1		1
5 plants†	16	126	9			1	
Total	54	463	30	4	3	1	1

* Chi square (1:1) for $R_1R_1r_1$:$R^{RAI}R^{RAI}r_1$ = 11.84** (df. = 2).

† Data from several small families combined.

‡ In all plants of classes (1) and (4) the small R_2 spot was present.

A more detailed classification was possible with the progenies from 6n S4025-*raimondii* with the genotype $R_1R_1R^{RAI}R^{RAI}$ (table 5). First, because of the presence of both red plant color alleles from *hirsutum* and large petal spot alleles from green *raimondii* it was possible to distinguish plants not only by their spot, as above, but also by their plant color. Three major classes occurred: 1) deep red body, no *raimondii* spot (a small spot was present in all 54 plants of this group, but as noted above this was caused by the independent A-genome allele R_2; fig. 1, left).

2) intermediate red body, *RAI* spot (fig. 1, lower center)—463 plants.

3) green body, deep *RAI* spot with pigmentation at the base of the androecium (fig. 1, right)—30 plants.

These three classes were expected from normal two by two disjunction of the four alleles R_1-R_1-R^{RAI}-R^{RAI} present in the hexaploid into three types of eggs: R_1R_1, R_1R^{RAI}, and $R^{RAI}R^{RAI}$.

Furthermore, advantage could be taken of the fact that both R_1 and R^{RAI} show dosage effect. Thus two R_1 alleles produced a much more intense leaf pigmentation than one R_1 alone; similarly two R^{RAI} alleles intensify spot and filament pigmentation as compared with one only. There was even a qualitative difference in that plants with two alleles showed red pigment in the fused base of the androecium, whereas those with one R^{RAI} did not (fig. 1). These distinctions made it possible to decide whether all progeny plants had regularly obtained two alleles from their hexaploid parent or whether irregularities in disjunction had occurred. The right side of table 5 reveals that exceptional offspring actually were obtained:

4) intermediate red body, no *RAI* spot; small R_2 spot present—4 plants,

5) green body, weaker *RAI* spot without pigmentation of the androecium base—3 plants. The last two classes supposedly resulted from gametes with only one allele, whereas the next two had three alleles instead:

6) deep red body, weaker *RAI* spot—1 plant,

7) intermediate red body, deep *RAI* spot without pigment at the base—1 plant.

Thus all the possible types deficient for the chromosome, or with an extra dose of it occurred but constituted together only 1.6% of the total.

Segregation on the male side was also tested in the two combinations 6n S5082-

FIGURE 1.—Flowers of genotypes $R_1R_1r_1$, $R_1R^{RAI}r_1$, $R^{RAI}R^{RAI}r_1$ (from left to right; smallest unit on ruler represents 1 mm).

TABLE 6

Segregation for petal spot in 6n G. hirsutum-raimondii *as male parent*
(*6n* G. hirsutum-thurberi × *6n* G. hirsutum-raimondii)

Male parent		Number plants		1163:134* chi square
Combination	Plant no.	*RAI* spot	No *RAI* spot	
6n *G. hirsutum* S5082-*raimondii*	1-209-A	89	8	0.45
	1-209-C	51	7	0.19
	4 plants†	68	7	0.08
6n *G. hirsutum* SM2-*raimondii*	4 plants†	42	7	0.83
Totals		250	29	1.55

* Comparison with totals from table 4.
† Data from several small families combined.

raimondii and 6n SM2-*raimondii*. As 4n × 6n crosses did not produce any mature seeds, 6n × 6n crosses had to be substituted and 6n *G. hirsutum-thurberi* which did not carry the *raimondii* spot were used as female testers. The results listed in table 6 were homogeneous and were similar to those obtained from the *hirsutum-raimondii* hexaploids as female parents (chi square 1.55; df. = 4).

Progeny tests

RHYNE (1951) has presented evidence that the *raimondii* spot gene and the R_1 factor are allelic and that all three alleles R_1, R^{RAI} and r_1 have distinct qualitative

TABLE 7

Progeny tests of the classes of offspring listed in table 5
(Crossed as ♀♀ with non-spotted tester)

Postulated genotype (class)		No. plants in test	Red plants		Green plants	
			RAI spot	No *RAI* spot	*RAI* spot	No *RAI* spot
$R_1R_1r_1$	(1)	2	0	191	0	63
R_1r_1	(4)	2	0	36	0	27
$R_1R^{RAI}r_1$	(2)	9	135	316	247	276
$R_1R^{RAI}R^{RAI}r_1$	(7)	1	19	2	29	1
$R^{RAI}R^{RAI}r_1$	(3)	2	0	0	363	57
$R^{RAI}r_1$	(5)	2	0	0	29	26

effects. But in order to ascertain whether the differences in intensity of pigmentation between the expected and exceptional classes in table 5 had a genotypic basis progeny tests were performed. Representatives from six of the seven classes listed in table 5 were crossed, as females, to *hirsutum* r_1r_1 and the results are presented in table 7. The plant in class 6, postulated to be $R_1R_1R^{RAI}r_1$, occurred only last season and progeny is not yet available.

The tested types are arranged in such a way in table 7 that those which differed quantitatively are listed together in pairs. The classes of the first two rows (designated as 1 and 4 above) differed in the intensity of their plant body pigmentation. Plants with intense pigmentation of the postulated genotype $R_1R_1r_1$ produced about three times as many red offspring as green (as may be expected from a trisomic with partial transmission) whereas the lighter plants had about equal numbers of red and green progeny which confirmed their genotype as R_1r_1. Classes 2 and 7, presented with their offspring in the next two rows, had similarly intermediate red body color but differed in the intensity of their *RAI* spot, filaments and androecium base. Their progenies differed distinctly in the frequencies of non-spotted types: these were rarely produced by the plant postulated to be $R_1R^{RAI}R^{RAI}r_1$ but of high frequency in the progenies from $R_1R^{RAI}r_1$ as would be expected. Classes 3 and 5, in the last two rows, had no plant anthocyanin but differed in the intensity of the *RAI* complex: $R^{RAI}R^{RAI}r_1$ produced less than one seventh spotless plants whereas $R^{RAI}r_1$ had about one half spotless offspring, as expected. It might be mentioned here that the low frequency of recessive offspring from $R^{RAI}R^{RAI}r_1$ might seem at first incompatible with the much higher frequency of recessives obtained from $R_1R_1r_1$. But in the latter type, in which all three chromosomes are of *G. hirsutum* origin (except for segments exchanged by crossing over) they conjugate and separate at random, whereas in $R^{RAI}R^{RAI}r_1$ preferential pairing between the two chromosomes from *G. raimondii* will increase the proportion of gametes with R^{RAI}. The detailed study of the effects of preferential pairing and of trisomic transmission in the offspring of allopolyploids must be postponed for a later date, however.

Linkage

G. hirsutum S5082 carries the linkage group r_1-*cl* for which crossover distances ranging from 13.9 to 18.5 units have been reported (HARLAND 1934; NEELY 1942).

The hexaploid with *G. raimondii* has normal (*Cl*) flowers with *RAI* spot like *G. raimondii* itself. Crosses between this hexaploid and *G. hirsutum* were made with S5082 as the *hirsutum* parent; from such crosses 160 *RAI* spotted and 24 non-spotted plants were obtained (table 4: rows 4 and 5). All 160 *RAI* spotted plants were non-cluster, all 24 non-spotted plants carried cluster.

Strain SM4 carries the gene yg_1 for yellow-green, which RHYNE (1955) suspected to be linked with r_1. The hexaploid combination with *G. raimondii* is fully green. Recombination occurred in the progeny obtained from crossing to SM4; of the 28 plants with *RAI* spot (table 4) 27 were green and 1 was yellow-green. The 6 plants without *RAI* spot were 5 yellow-green and 1 green.

6n G. hirsutum-thurberi

For a comparison with the results from the *G. hirsutum-raimondii* hexaploids just described a 6n *G. hirsutum-thurberi* combination was also studied. As anthocyanin is largely limited in *G. thurberi* to a weak and recessive spot the dominant R_1 marker of *hirsutum* strain S4025 was used. Hexaploid hybrids with *thurberi* were produced and backcrossed to r_1r_1 *hirsutum* (table 8A). Thus far only crosses with the hexaploids as female parents are available. The same hexaploids were also crossed, as females, with 6n *G. hirsutum-raimondii* lines which carried r_1r_1 and $R^{RAI}R^{RAI}$, but no plant color R_1 (table 8B). (These latter crosses were already listed earlier, in table 6, but there from the standpoint of segregation for *RAI* in the male parent, while here segregation for R_1 in the female parent is of interest.)

The data from several plants and from the two types of crosses (table 8) were homogeneous (chi square = 7.85; df. = 4), but differed considerably from those obtained with the hexaploids containing *G. raimondii*. Thus, from 6n *G. hirsutum* S4025-*raimondii* 84 plants had been obtained out of a total of 547 (table 5) which were not intermediate, disregarding the exceptional classes. On the other hand, the *G. hirsutum* S4025-*thurberi* hexaploids gave only 25 non-intermediate plants out of 613 (table 8) which is very much less (chi square = 43.17**).

G. thurberi has no dominant allele at the R_1 locus and, therefore, it was not possible to recognize exceptional classes comparable to those of table 5. On the other hand, two exceptional plants of a different type were found. It may be recalled that the *hirsutum* parent, S4025, carried R_2R_2 in its A chromosomes; of course, no segregation was expected in the hexaploids possessing only two identical A genomes. None was observed among the progeny of 6n S4025–*raimondii*, but only the 58 plants without R^{RAI} could be scored for R_2 because the *RAI* spot would have obscured any absence of the smaller R_2 spot in all others. But there was no large spot to conceal R_2 in the entire progeny from 6n S4025–*thurberi* × *hirsutum* r_1r_2 (334 plants; table 8A) and two exceptional plants were found without petal spots in the intermediate class. These will be considered later.

DISCUSSION

The hexaploids used in this study were not true allopolyploids with respect to their D genomes but rather segmental allopolyploids, using the terminology of STEBBINS (1947). With homogenetic pairing (WADDINGTON 1939) between chromosomes derived from the same parental species, which is characteristic of true allopoly-

TABLE 8

Segregation for plant color in 6n G. hirsutum-thurberi

Plant no.	Progeny plant color			9:588:16 chi square
	Red	Intermediate	Green	
A. 6n G. hirsutum *S4025*-thurberi × hirsutum r_1r_1				
1-214-B	2	103	4	0.59
1-214-D	4	135	3	1.89
3 plants*	0	83	0	3.53
B. 6n G. hirsutum *S4025*-thurberi × *6n* hirsutum (r_1r_1)-raimondii				
1-214-D	2	188	5	0.27
5 plants*	1	79	4	1.57
Totals	9	588	16	7.85

* Data from several small families combined.

ploids, only gametes of one type would be formed by each of the polyploids (R_1R^{RAI}, r_1R^{RAI} or R_1R^{THU}, respectively) but none like R_1R_1, $R^{RAI}R^{RAI}$, etc. Presence of the latter indicates that heterogenetic associations occur at meiosis. The extent of the associations between the chromosomes of different species is more difficult to evaluate. It would seem that conjugation between New World and *raimondii* chromosomes is far from random, because the gametic ratio observed was 1163:134 or 8.68:1 (table 4) instead of a random 5:1. By the same token, 6n *G. hirsutum-thurberi* gave even a poorer fit to the random ratio, namely 9:588:16 or 1:65.33:1.78 (table 8) instead of 1:4:1. These observed ratios could have been effected by partially homogenetic pairing of chromosomes from the same stock together with a certain amount of association between chromosomes from different species. Such a conclusion, however, would be based on the assumption that little selective elimination of the non-intermediate types occurred at post-meiotic stages. However, male and female gametic elimination certainly was considerable, as stated earlier, and zygotes were also eliminated at all seed and seedling stages; the question arises whether all this sterility was at random as far as the different alleles at the R_1 locus are concerned. No direct evidence is obtainable, of course, as no progeny can be raised from abortive gametes and zygotes. The problem has to be attacked indirectly.

First, reciprocal crosses may be compared and that has been done for 6n *G. hirsutum-raimondii* (cf. tables 4 and 6). The results were similar. Since it is unlikely that discrimination against pollen and eggs of a certain genotype is the same because of their entirely different conditions one might infer that gametic elimination is not significant. Secondly, one may compare the sizes of two non-intermediate classes, i.e., those derived from gametes R_1R_1 and $R^{RAI}R^{RAI}$ or R_1R_1 and $R^{THU}R^{THU}$ in those crosses in which both could be separated from the intermediates. In table 5 more plants came from R_1R_1 than from $R^{RAI}R^{RAI}$ gametes; the difference is below the 1% level of probability and suggests that offspring carrying the two *hirsutum*

alleles R_1R_1 were obtained somewhat more often than offspring with two alleles from *raimondii* ($R^{RAI}R^{RAI}$). It cannot be decided whether elimination took place at the gametic or zygotic stages. The observation is suggestive of the discrimination which STEPHENS (1949) found in backcrosses of F_1 hybrids between *Gossypium barbadense* and *hirsutum* and their parental species. There occurred a tangible deviation from a 1:1 ratio which tended to favor the alleles derived from the recurrent parent.

The difference between R_1R_1 and $R^{THU}R^{THU}$ classes (table 8) was in the opposite direction, but non-significant (chi square = 3.90, df. = 4). Because of the rarity of non-intermediate segregates from 6n *hirsutum-thurberi* very large populations are needed to establish whether any significant discrimination occurs.

From the above it appears that selective elimination is not very large whereas the difference in segregation between the hexaploids involving *G. raimondii* and *G. thurberi* is of a much higher order of magnitude. This difference may appear quite surprising in view of the fact that the chromosomes of *G. raimondii* and *thurberi* are homologous to the extent that in a diploid hybrid they are capable of forming sufficient chiasmata to be held together in pairs at metaphase. Of 21 pollen mother cells viewed at metaphase in acetocarmine squashes 19 had formed 13 pairs, while 12 pairs plus 2 univalents appeared in only 2 cells; the hybrid was very fertile (BOZA BARDUCCI and MADOO 1941). Similarly, triploid hybrids between New World cottons and both *G. raimondii* and *thurberi* show good conjugation of their D genome chromosomes (SKOVSTED 1937; BOZA BARDUCCI and MADOO 1941; BEASLEY 1942; and others). Cytological observations under our conditions on the diploid and triploid hybrids agree (unpublished). Evidence of some structural differentiation between D chromosomes was obtained, however, by BEASLEY (1942) who saw bridges at anaphase of *G. hirsutum-thurberi* triploid hybrids, although these may have been attenuation caused by phenomena other than inversion crossovers. More recently, MENZEL (1955) discovered in hybrids with cytologically distinguishable chromosomes that the pairing affinities between the right arms of the *G. raimondii* and *G. hirsutum* number 1 chromosomes were weaker than between the homologous *G. hirsutum* arms. But detailed cytological studies comparing both *G. thurberi* and *raimondii* chromosomes are as yet lacking.

One has also to consider the possibility that some of the "segregation" may be due to loss rather than to recombination of chromosomes. Gametes with missing chromosomes may function as the change in chromosome balance wrought by such a loss is not serious in polyploids and many examples are on record. One might recall the case of MÜNTZING (1935) who found a reduced chromosome number in *Nicotiana digluta* after several generations of selfing. In Gossypium also sectors have been found on artificial polyploids which had fewer chromosomes than the rest of the plant; often the aberrant sector was limited to a few or perhaps only one meiocyte (MENZEL and BROWN 1952; SARVELLA unpublished). Gametes resulting from such sectors would be lacking certain chromosomes. Loss might also occur during meiosis itself through an unbalanced distribution of chromosomes or lagging. The frequency of occurrence of deficient offspring was tested in two cases, namely, for the R_1 chromosome itself among the progeny of 6n *G. hirsutum-raimondii*

and found to be rather rare (table 5, classes 4 and 5). It has also been obtained for the progeny from 6n *G. hirsutum-thurberi* for the R_2 locus on A chromosomes which should form bivalents regularly and was found in only 2 out of 334 plants. These exceptions, of course, might have been caused by mutation or intergenomic A-D crossing over as well as by chromosome loss. Significant in this connection is the observation that one of the two spotless plants showed a unique scalloping of the leaf lobes while the other did not differ in this respect from the regular sibs with R_2 petal spots.

Pending further evidence on the amount of postmeiotic selective elimination it appears that the *G. thurberi* homologues bearing R^{THU} show much stronger homogenetic association in the hexaploid with *G. hirsutum* than the members of the *G. raimondii* pair. Thus, the genetic segregation of allopolyploids may reveal differences between the chromosomes of the parental species which cannot be easily detected by a cytological inspection of F_1 hybrids. This interpretation is based on DARLINGTON's (1937) theory of differential affinity which postulated that in the same nucleus chromosomes which are similar throughout their length are more likely to be associated than chromosomes which are homologous only in part. It would follow that there exists a quantitative relationship between the degree of similarity and the extent of preferential association of chromosomes.

To the taxonomist of the genus Gossypium these results do not come as a surprise. While *G. raimondii* and, of course, *G. hirsutum* are typical members of the genus, *G. thurberi* is a specialized type from the northern edge of the distribution of the American Gossypiums. Its strongly angular stem cross sections, shallowly cupped flowers and triangular and almost glabrous seed distinguish it from the rest of the genus. It also has a pale yellow flavonol pigment in its petals which occurs in no appreciable amount in any other Gossypium (STEPHENS personal communication). In GRAY's (1855) original description this species was placed in the separate genus Thurberia. Though its position in Gossypium is now assured by the cytotaxonomic work of SKOVSTED (1937), WEBBER (1939) and especially BEASLEY (1942) the present results suggest that at least one of its chromosome pairs has become considerably differentiated.

On the other hand, *G. raimondii* had been postulated on morphological grounds to be more closely related to the cultivated New World cottons than other wild Americans by STEPHENS (1944) and by HUTCHINSON, STEPHENS and DODDS (1945). The genetical behavior of the hexaploids described here parallels the morphological features of *G. thurberi* and *G. raimondii*.

This investigation is concerned with merely one locus. Studies are in progress which are devoted to the behavior of marker genes on other chromosomes and in other polyploid hybrids in order to see whether different chromosomes in the same species combination give similar segregation frequencies and to what extent the segregation pattern in the various combinations also follow taxonomic relationships.

SUMMARY

Segregation in two synthetic allopolyploids was intermediate between that expected from random chromosome behavior (5:1 gametic output) and a true allopolyploid

(∞:0). Segregation for the *RAI*-spot in 6n New World-*raimondii* scored in the backcross was 1163 *RAI*:134 non-*RAI* (8.68:1) with non-significant differences between hexaploids synthesized from different New World lines (one exception). In contrast, a New World-*thurberi* hexaploid gave a 604:9 (= 67.1:1) ratio for corresponding alleles.

These allopolyploids, as is usual for newly synthesized polyploids, were only partially fertile and whether the observed deviations from 5:1 were a reflection of meiotic behavior or due to post-meiotic selection had to be considered. Only indirect attacks were possible, by use of reciprocal crosses or comparison of the *AA* and *aa* classes where both could be distinguished in the progeny of the *AAaa* model. Indications were that some post-meiotic selection occurs, but of much smaller magnitude than the difference between the two types of hexaploids.

There was also some evidence for irregular meiotic disjunction which produced exceptional gametes of the types *A*, *a*, *AAa*, and *Aaa*, but they were very infrequent. Similarly, irregularities were rare with respect to a factor located in a chromosome for which the hexaploid was disomic.

The narrower segregation ratio in 6n New World-*raimondii* as compared with 6n New World-*thurberi* may be indicative of a closer taxonomic relationship between the New World cottons and *G. raimondii* than between the former and *G. thurberi*.

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IMMUNOGENETIC STUDIES OF PSEUDOALLELISM IN DROSOPHILA MELANOGASTER. II. ANTIGENIC EFFECTS OF THE VERMILION PSEUDOALLELES[1]

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A PREVIOUS study of the antigenic effects of the lozenge pseudoalleles in *D. melanogaster* (CHOVNICK and FOX 1953) yielded results sufficiently fruitful to encourage a similar study of the vermilion pseudoalleles. Extensive studies of the genetics and physiology of the vermilion mutants have been performed by M. M. GREEN (1949, 1952, 1954) and by P. B. SHAPARD (1954). All such mutants are recessive and are characterized by the almost complete absence of brown pigment in the eye. All accumulate non-protein tryptophane at a much higher level than wild type, all are non-autonomous in gynandromorphs with v^+, and all produce brown pigment when supplied with exogenous kynurenine or formylkynurenine. They presumably suffer from the inability to convert tryptophane into formylkynurenine, which serves as the precursor of kynurenine in the synthesis of brown pigment. In spite of these similarities, they may be separated into two physiological categories on the basis of their phenotypes in the presence of the non-allelic, linked suppressor mutants (*su*^{2}-*s* and *su*^{9}-*v*). Some, as exemplified by v^1, exhibit the wild type phenotype in the presence of either suppressor and are collectively designated as v^s, while others, as exemplified by v^{36f}, exhibit an unaltered phenotype and are collectively designated as v^u. The distinction is reported to be unequivocal, and accumulation of non-protein tryptophane is also suppressed in v^s mutants but not in v^u. A parallel separation is afforded by an additional physiological criterion: partial starvation of larvae results in the production of brown pigment in all v^s mutants but not in v^u. Finally, the separation of the mutants into two classes on the basis of physiology is apparently paralleled by a difference in mutational origin. Among the mutants studied, all of those which were X-ray induced were unsuppressed, while the spontaneous mutants (including v^1 and v^{36f}) fell into both physiological categories.

The pseudoallelism of vermilion mutants has been demonstrated in an elegant experiment performed by GREEN (1955). Crossing over between v^1 and v^{36f} was observed with low frequency, thus suggesting that they are mutants at two closely linked loci bearing the following relationships to the raspberry (1–32.8) and miniature (1–36.1) loci on the X chromosome:

$$\underline{ras\ 0.2 v^1\ v^{36f} \qquad 3.1 \qquad m}\ .$$

In addition, these mutants exhibit the type of position effect characteristic of pseudoalleles in Drosophila: the "trans" heterozygote (v^1/v^{36f}) exhibits the mutant phenotype, while the "cis" heterozygote ($v^1 v^{36f}/+$) is wild type.

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On the basis of the demonstration of pseudoallelism, and on the basis of the observations referred to above, GREEN suggests that all v^s mutants are alleles of v^1 and that all v^w mutants are alleles of v^{36f}. He further suggests that the two loci are also distinguishable on the basis of mutability, *i.e.* that all induced mutations involve the v^{36f} locus either as "point" mutations at that locus alone or as small rearrangements or "point" mutations at both loci. It should be noted, however, that definitive crossover tests of the various mutants with v^1 and v^{36f} have not been reported.

From the point of view of theory, the implication of these suggestions is that the two vermilion loci are separable not only on the basis of crossing over, but also on the basis of their physiology and mutability. Extended to pseudoallelism in general and to the theory of the gene, the implication is that the gene defined as a breeding unit displays physiological and mutational unity as well (GREEN 1955).

The previous paper in this series (CHOVNICK and FOX 1953) suggested that an operational attitude toward the problems of pseudoallelism would insist on no unique relationship between the transmissional behavior of the genetic material and its physiological or mutational attributes. The apparent contradiction posed by pseudoallelic mutants, namely the fact that they are transmitted as non-alleles but exhibit the physiological properties of alleles, springs from such an insistence (GOLDSCHMIDT 1951) but disappears when viewed operationally. In the present instance, operationism would restrain the contrary expectation that a series of mutants which are transmitted as alleles at one pseudoallelic locus need be similarly separable from mutants at the other pseudoallelic loci on the basis of physiological or mutational criteria.

The present paper contains evidence bearing on this point. It also discusses the question of the origin of pseudoalleles, the mechanisms of their physiological action, and the problem of the position effect which they exhibit in light of the antigenic effects of the vermilion pseudoalleles.

STOCKS

Three vermilion mutants were used in this investigation: v^1, v^{36f}, and v^{48a}. The first two have been adequately described (BRIDGES and BREHME 1944); v^{48a} first appeared in a single male from a mating of an X-rayed male with an unirradiated attached-X female, and possesses apparently normal salivary chromosomes (FOX 1948). It is characterized by the production of an antigenic component ("antigen 2") which is not produced by either of two different wild alleles (FOX 1949b; FOX and WHITE 1953). GREEN (1953) reports that it is a v^w mutant, and that no crossovers between it and v^1 were observed among 40,000 offspring (GREEN 1954 and personal communication).

Mutant stocks containing v^1, v^{36f}, v^{48a}, and v^1v^{36f} were kindly provided by M. M. GREEN. For purposes of antigenic analysis it was necessary that the mutants be placed on a coisogenic background. The background chosen was that of the isogenic Oregon R (Series I) stock used in the analysis of the lozenge pseudoalleles (CHOVNICK and FOX 1953). This stock had originally been rendered isogenic by JACK SCHULTZ and had been maintained thereafter through 112 generations of brother-sister matings at the time of the present derivation.

In preparation for the derivation, each of the single mutants and the double mutant combination were inserted into a marked X chromosome: *y ras*^{2} *v m f*. The derivation itself was accomplished by inserting the vermilion segment of each of the X chromosomes mentioned above into the X chromosome of the Oregon R stock, and by replacing autosomal chromosomes with those of Oregon R. In detail, *y ras*^{2} *v m f* females were crossed with *In(2L)Cy*, *Cy*, *In(2R)Cy*, *cn*^{2}/*In(2LR)Pm*, *ds*^{33k}; H/*In(3R)Mo*, *Sb sr* males, producing *y ras*^{2} *v m f*/*Y*; *Cy*/+; *Sb*/+ males which were mated to females from the isogenic Oregon R stock. Females from this mating having the genotype *y ras*^{2} *v m f*/+; *Cy*/+; *Sb*/+ were mated in turn with Oregon R males. Crossover male offspring of either *y ras*^{2} *v*/Y or *v m f*/Y genotype were recovered. Using the former as an example, one such male was crossed with Oregon R females, yielding *y ras*^{2} *v*/+ females which were mated with Oregon R males. Crossover *v*/Y males were recovered. One such male was mated with Oregon R females, yielding *v*/+ daughters. These were mated with their father to give *v*/Y and *v*/*v* males and females, which were then used to establish isogenic vermilion stocks.

Four such stocks were established; three single-mutant stocks (v^1, v^{36f}, and v^{48a}), and the double mutant (v^1v^{36f}). Each of these stocks possessed Y, second, and third chromosomes of isogenic Oregon R origin, and a vermilion segment inserted into an Oregon R X chromosome. Together with the Oregon R wild stock, they made available five coisogenic stocks differing with respect to the vermilion pseudoalleles. From these, equal numbers of homozygous females and hemizygous males could be collected as desired. Heterozygous females were produced as needed by crosses between the stocks.

The following qualifications of coisogenicity should be mentioned: (1) Aside from the differences with respect to the vermilion loci, the X chromosomes of the stocks may differ with respect to a segment no longer than 0.2 crossover units to the left and 3.1 units to the right of the vermilions. (2) Heterogeneity of autosomes (2 and 3) may have resulted from crossing over between Oregon R chromosomes and "marker" chromosomes during the derivation, in spite of the inversions present in the latter. (3) The fourth chromosome was not controlled during the derivation. Repeated outcross to Oregon R renders it probable, however, that the mutant stocks possess Oregon R fourth chromosomes. (4) Spontaneous mutation, without visible effect, could not be controlled during or after the derivation.

RESULTS

Suppression and larval starvation tests

To demonstrate that the mutants in our stocks behaved the same as those tested by GREEN, suppression tests were performed with *su*^{g}-*v* (1−0.0+). The method used was essentially the same as that used by GREEN (1952). In large experiments, *su*^{g}-*v* v^1 males were wild in phenotype, while newly emerged *su*^{g}-*v* v^{36f} and *su*^{g}-*v* v^{48a} males were vermilion. Thus, the mutants behave the same in our stocks as in GREEN's: v^1 is suppressed, and v^{36f} and v^{48a} are unsuppressed. In addition, it was observed that the double mutant combination, v^1v^{36f}, is unsuppressed.

The results reported below place v^{48a} in an important position, and an additional check was performed by means of the larval starvation test. Again, the method used

TABLE 1

Allelism of v^{48a}

Mating	Number of offspring	Non-vermilion offspring
ras^2 v^{36f} *m*/v^{48a}; *Cy*/+; *Sb*/+ × *ras*^{2} v^{36f} *m*/Y	78,934	1 *ras*, *Sb* 1 *ras*
ras^2 v^1 *m*/v^{48a}; *Cy*/+; *Sb*/+ × *ras*^{2} v^1 *m*/Y	32,734	0
ras^2 v^1 *m*/v^{48a}; *Cy*/+; *Ubx*/+ × *ras*^{2} v^1 *m*/Y*	40,000	0

* Data from GREEN (1954 and personal communication).

was the same as that used by GREEN (1954). Confirming his report, there is no production of brown pigment when v^{48a} is subjected to partial larval starvation.

The allelic status of v^{48a}

The test of allelism of v^{48a} with v^{36f} was performed by mating previously prepared *ras*^{2} v^{36f} *m*; *Cy*/*Pm*, *ds*^{33k}; *H*/*In*(3*R*)*Mo*, *Sb sr* females with v^{48a} males, to yield *ras*^{2} v^{36f} *m*/v^{48a}; *Cy*/+; *In*(3*R*)*Mo*, *Sb sr*/+ females. These were then crossed with *ras*^{2} v^{36f} *m* males and the offspring were counted and classified. Crossovers between v^{36f} and v^{48a} would yield non-vermilion offspring; miniature if v^{48a} were to the right of v^{36f}, and raspberry if it were to the left of v^{36f}. The presence of inversions in the second and third chromosomes of the heterozygous females enhances crossing over between the X chromosomes; *ras*^{2} and *m* guard against mistaking spontaneous mutants for crossovers; *Cy* and *Sb*, along with *ras*^{2} and *m*, guard against contamination. Similar crosses were performed using v^1 instead of v^{36f} to test the allelism of v^{48a} with v^1. The results of these tests, together with similar results reported by GREEN, are contained in table 1.

In the tests with v^{36f}, two raspberry males were observed among 78,934 counted offspring. These were demonstrated to be genotypically *ras*^{2} by the results of matings with *ras*^{2} *v m* and wild females. They show that v^{48a} is pseudoallelic to v^{36f}, occupying a locus to the left of that of v^{36f}. In the tests for allelism involving v^{48a} and v^1, no crossovers were recovered among 32,734 offspring. If the results of GREEN are added to ours, no crossovers have been observed among approximately 73,000 offspring. There is thus no evidence that v^{48a} is anything but an allele of v^1, and the fact that they are both located to the left of v^{36f} is in agreement with this conclusion.

Immunological analysis

Methods used in the preparation of antigens and antisera and in the performance of immunological tests are given in previous publications (CHOVNICK and FOX 1953; FOX 1949b; FOX and WHITE 1953; FOX 1949a). Antigens were prepared by homogenizing approximately equal numbers of males and females of each of the coisogenic stocks (Oregon R, v^1, v^{36f}, v^{48a}, and v^1v^{36f}) and females of each of the two heterozygous genotypes, v^1/v^{36f} and $v^1v^{36f}/+$. Rabbit antisera were prepared to each of these antigenic preparations. Antisera to each of the first five antigenic preparations were absorbed with each of these five antigens and each absorption was then tested by means of modified precipitin-ring tests with all seven antigens. Antisera to the two hetero-

TABLE 2

Analysis of anti-v^1 serum

Absorbed by	Tested with						
	Ore-R	v^1	v^{36f}	v^{48a}	v^1v^{36f}	v^1/v^{36f}	$v^1v^{36f}/+$
Unabsorbed	+	+	+	+	+	+	+
Oregon R	−	+	+	+	+	+	+
v^1	−	−	−	−	−	−	−
v^{36f}	−	−	−	−	−	−	−
v^{48a}	−	−	−	−	−	−	−
v^1v^{36f}	−	−	−	−	−	−	−

Antiserum, normal serum, and antigen controls all negative.

zygotes were absorbed by each of the seven antigens and each absorption was then tested with all seven antigens. Antigen, antiserum, and normal serum controls were included in all tests. The tests were semi-quantitative in nature, similar to those presented in previous publications, although only qualitative summaries of the results are given: the symbol + indicates a definite reaction of any strength, while − indicates the lack of reaction. The differences reported are distinctly qualitative, and in each case absorptions with excess heterologous antigen did not inhibit the reactions with homologous antigen.

The results obtained with anti-v^1 serum are summarized in table 2. Before absorption, such serum reacts with all antigenic preparations with equal titer. Most of the antigens are therefore similar in all genotypes; these will be designated residual antigens. Following absorption with Oregon R antigens, differences among the genotypes begin to appear. At the proper level, such absorption removes from the serum all antibodies against components which Oregon R shares with v^1, but the serum still reacts with the antigens of the six mutant genotypes. This indicates that v^1 possesses an antigenic component (simple or complex) which is not possessed by Oregon R but which is also present in the other mutant genotypes.

The specificity of the serum is made evident by its exhaustion by absorption with v^1. It is similarly exhausted by absorption with each of the other mutant genotypes, indicating that v^1 possesses no antigenic components not also possessed by these other genotypes. Thus, anti-v^1 serum evidently contains antibodies specific to an antigenic component common to all six of the vermilion genotypes but not present in Oregon R. This component will be referred to as antigen V-1.

Table 3 contains the results obtained with anti-v^{36f} serum. The reactions with unabsorbed serum confirm the existence of residual antigens. The conclusion reached above that the vermilion genotypes contain at least one antigenic component (V-1) not possessed by Oregon R is also confirmed, since absorption by Oregon R inactivates the serum only with respect to Oregon R. In addition, absorption by v^1 yields results indicating the presence of anti-v^{36f} serum of antibodies to an antigenic component other than V-1, common to v^{36f}, v^{48a}, v^1v^{36f}, and the heterozygotes, but not present in either Oregon R or v^1. Thus, a second antigenic component, V-2, is identified.

TABLE 3

Analysis of anti-v^{36f}*, anti-*v^{48a} *(Type 1), anti-*v^1/v^{36f}*, and anti-*$v^1v^{36f}/+$ *sera*

Absorbed by	Tested with						
	Ore-R	v^1	v^{36f}	v^{48a}	v^1v^{36f}	v^1/v^{36f}	$v^1v^{36f}/+$
Unabsorbed	+	+	+	+	+	+	+
Oregon R	−	+	+	+	+	+	+
v^1	−	−	+	+	+	+	+
v^{36f}	−	−	−	−	−	−	−
v^{48a}	−	−	−	−	−	−	−
v^1v^{36f}	−	−	−	−	−	−	−

Antiserum, normal serum, and antigen controls all negative.

It should be remarked that not all antisera exhibit such specificity. Thus, among anti-v^1 sera some are non-specific, but those which exhibit specificity yield the results contained in table 2. Such experiences are frequent when working with complex antigenic mixtures (IRWIN 1949; FOX and WHITE 1953), and the occurrence of non-specific antisera does not contradict the existence of specificity when it is demonstrated. Indeed, it is frequently observed that an antiserum may contain antibodies to some but not all of the components in an antigenic mixture, and that different sera may be specific for different components.

Such is the case among anti-v^{48a} sera. Those which exhibit specificity are of two types, designated 1 and 2. Table 3 contains the results obtained with anti-v^{48a} sera of the first type; the specificities exhibited by this type of serum are identical with those exhibited by anti-v^{36f} serum. Such antiserum thus yields confirmatory evidence of the existence of antigens V-1 and V-2 as well as residual antigens.

The second type of anti-v^{48a} serum yields evidence of an additional component, but contains no antibodies to antigens V-1 or V-2 (Table 4). This is indicated by the fact that absorption with Oregon R completely inactivates the serum, while absorption by v^1 or v^{36f} does not inactivate it with respect to v^{48a} and v^1v^{36f}. Apparently v^{48a} possesses an antigenic component, designated V-3, which is also present in v^1v^{36f} and in Oregon R.

The conclusion that v^{48a} and v^1v^{36f} possess antigen V-3 is based on two observations;

TABLE 4

Analysis of anti-v^{48a} *(Type 2) and anti-*v^1v^{36f} *sera*

Absorbed by	Tested with						
	Ore-R	v^1	v^{36f}	v^{48a}	v^1v^{36f}	v^1/v^{36f}	$v^1v^{36f}/+$
Unabsorbed	+	+	+	+	+	+	+
Oregon R	−	−	−	−	−	−	−
v^1	−	−	−	+	+	−	−
v^{36f}	−	−	−	+	+	−	−
v^{48a}	−	−	−	−	−	−	−
v^1v^{36f}	−	−	−	−	−	−	−

Antiserum, normal serum, and antigen controls all negative.

TABLE 5

Analysis of anti-Oregon R serum

Absorbed by	Tested with						
	Ore-R	v^1	v^{36f}	v^{48a}	v^1v^{36f}	v^1/v^{36f}	$v^1v^{36f}/+$
Unabsorbed	+	+	+	+	+	+	+
Oregon R	−	−	−	−	−	−	−
v^1	−	−	−	−	−	−	−
v^{36f}	−	−	−	−	−	−	−
v^{48a}	−	−	−	−	−	−	−
v^1v^{36f}	−	−	−	−	−	−	−

Antiserum, normal serum, and antigen controls all negative.

both completely inactivate the serum when used in absorptions, and both react with the serum after it has been absorbed by v^1 or v^{36f}. Oregon R, on the other hand, exhausts the serum when used in absorptions but does not react with serum previously absorbed with v^1 or v^{36f}. Oregon R, in other words, possesses V-3 specificity, but in a form incapable of precipitating V-3 antibodies even though uniting with them. The result, in immunological terminology, is inhibition.

Confirmation of the existence of antigen V-3 is obtained from the analysis of anti-v^1v^{36f} serum (table 4). Such antiserum reacts in a manner identical with that of Type 2 anti-v^{48a} sera. V-3 antibodies are present, and they are precipitated by the corresponding antigen in v^{48a} and v^1v^{36f}. Oregon R, however, inhibits these antibodies without precipitation; V-3 specificity is therefore present, but in a different form than in v^{48a} and v^1v^{36f}.

Further information concerning the nature of V-3 in Oregon R is obtained from the analysis of anti-Oregon R serum (table 5). None of several such antisera have contained antibodies to antigens other than residual antigens. Antibodies to antigens V-1 and V-2 are not expected, since these antigens have been demonstrated not to be present in Oregon R. Beyond this, there is no evidence that Oregon R contains any other specific antigenic components. Antigen V-3, as it is present in Oregon R, is therefore not only incapable of precipitating homologous antibody, but is also incapable of inducing antibody formation. While it is present in the form of a complete antigen in v^{48a} and v^1v^{36f}, it behaves like a hapten in Oregon R.

LANDSTEINER (1945) defines haptens as "... specific protein-free substances, although reactive in vitro, induced no, or only slight antibody response. For serologically active substances of this sort, in contradistinction to the protein antigens which possesses both properties, the term hapten has been proposed ..." The serological behavior of V-3 in Oregon R corresponds with this definition. Preliminary tests indicate that, in Oregon R, V-3 is heat-stable and nondialyzable. These tests have not been extensive, however, and further evidence concerning the chemical nature of the V-3 substance in Oregon R is not yet available.

Cis and trans heterozygotes for v^1 and v^{36f} have thus far been demonstrated both to possess antigens V-1 and V-2. Therefore, no position effect has yet been found. Differences between the two heterozygous genotypes may exist, however, with

TABLE 6

Summary of antigenic distribution

Genotype	Antigenic Components			
	V-1	V-2	V-3	Residual
Oregon R	−	−	Haptenic	+
v^1	+	−	−	+
v^{36f}	+	+	−	+
v^{48a}	+	+	+	+
v^1v^{36f}	+	+	+	+
v^1/v^{36f}	+	+	−	+
$v^1v^{36f}/+$	+	+	−	+

respect to antigens not possessed by Oregon R or the homozygous and hemizygous mutants. Such differences would be disclosed in the analysis of antisera against the heterozygotes (anti-v^1/v^{36f} and anti-$v^1v^{36f}/+$). Both kinds of antisera react in the manner contained in table 3. The reactions summarized therein are indicative of the presence of antigens V-1 and V-2, as well as residual antigens, in both kinds of heterozygote. No differences between the two heterozygotes are indicated, and therefore no position effect on antigens.

The antigenic relationships of the analyzed genotypes are summarized in table 6.

DISCUSSION

As was the case with respect to the antigenic effects of the lozenge pseudoalleles, the results of the present investigation are consistent with the suggestion that pseudoalleles arise by a process of genic replication along the chromosome, accompanied or followed by complementary physiological differentiation (BRIDGES 1935; LEWIS 1951). The similarities in antigenic effects which have been encountered among pseudoallelic mutants suggest duplication; the differences suggest partial differentiation.

It was suggested, in connection with the lozenge analysis, that the physiological differentiation of pseudoalleles could take three forms: (1) With slight changes in substrate specificity pseudoallelic loci could become associated with successive steps in a sequence of reactions. This suggestion is incorporated in the physiological hypothesis applied to pseudoallelism by LEWIS (1951) and by PONTECORVO (1950). (2) Pseudoallelic loci could be modified in such a manner as to convert the same substrate or substrates into one or more products, similar in some respects and different in others. Such differentiation would reflect more directly the duplicate origin of the loci than would differentiation of the first type. (3) Each locus could be concerned with the transformation of two or more substrates, and differentiation could take the form of a gradual shift in "substrate spectrum." As in the previous case, such differentiation would be an easy consequence of origin by duplication. The antigenic effects of the lozenge pseudoalleles were such as to be explainable by means of simple models based on any of these three suggestions. In the present instance, the complexity of the antigenic effects render it extremely unlikely that they are produced by

the association of the two vermilion loci with successive steps in a linear sequence of reactions. At any rate, we have not been able to construct a model of this sort that would be consistent with the results without numerous *ad hoc* assumptions. Simple models based on the second and third suggestions will explain the present results. Since data are not yet available to distinguish between these latter models, it does not seem profitable to specify them in greater detail at the present time. Antigenic analysis of the unexamined genotypes should provide more definitive information.

However, the inconsistency of the present data with the possibility that the two vermilion loci are concerned with successive steps in a chain of reactions deserves additional comment. As indicated, that possibility is an integral part of the LEWIS hypothesis of the physiology of pseudoallelism. A second feature of the hypothesis is that the action of pseudoallelic loci is restricted to the immediate vicinity of the chromosome. Since the antigenic effects of the lozenge pseudoalleles suggested interallelic interaction at either or both of two loci (lz^{46} and lz^{BS}), immunogenetic studies of pseudoallelism have yielded evidence difficult to reconcile with both aspects of the hypothesis. It is entirely possible, of course, that the processes of antigen formation are entirely unrelated to those concerned with the morphological and pigmentary effects that form the basis of the hypothesis. Little is known about the physiological mechanisms responsible for the morphological effects of the lozenge mutants. CHOVNICK (personal communication) suggests that an affect on the development of hypodermal derivatives may be involved, but no obvious connection with the antigenic effects exists. No gross effects on amino acid metabolism are detectable by means of paper chromatography of free amino acids. (FOX 1954). It has been suggested (NOLTE 1952) that the effects of eye color mutants are associated with protein metabolism, but the connection with antigenic effect may nevertheless be remote. The tryptophane content of proteins in vermilion is not detectably different from that of wild (GREEN 1949) in spite of the antigenic differences. The two models outlined above suggest either that tryptophane is the common substrate for the production of eye pigment and antigenic effect but that these are produced along different pathways, or that tryptophane is only one of several substrates utilized by the vermilion loci and not the substrate of antigenic effect.

An additional circumstance suggests that the antigenic effects of pseudoalleles are associated with different processes than the morphological effects, namely the failure to demonstrate a position effect on antigenic specificity in heterozygotes of both the lozenge and the vermilion mutants. These observations demonstrate that position effect is not a necessary physiological property of "position pseudoalleles" at all phenotypic levels. The suggestion of physiological unrelatedness of the antigenic and morphological effects does not completely resolve the difficulties for the LEWIS hypothesis, however, for a study of the effects of the lozenge pseudoalleles on tarsal claws, where a position effect is observed, has yielded evidence posing similar difficulties (CHOVNICK personal communication).

Considering the antigenic effects of the vermilion pseudoalleles in greater detail, it is to be noted that more than one antigen may be associated with a single gene. A similar observation was made in connection with the lozenge mutants, and recently in connection with the antigens determined by alleles at the H-2 locus in the mouse

(HOECKER, COUNCE, and SMITH 1954). The bearing of such observations on questions regarding the Rh and other similar systems in man and other species will be the subject of a separate communication. In this connection, the antigenic component previously (FOX 1949b; FOX and WHITE 1953) associated with v^{48a} and designated "antigen 2" is now shown to consist of at least three different components (V-1, V-2, and V-3). This resolution does not affect any previous conclusions. It should also be noted that antigen V-3 is present in v^{48a} and $v^{1}v^{36f}$, but not in v^{1} or v^{36f}. Thus, the double mutant is capable of producing an antigenic component which is not produced by either of its mutant genes separately, but which is produced by a different mutant at one of the two loci. In the double mutant the effect must therefore be the result of interaction of the two mutant genes. Since this is the case, V-3 must be added to the list of antigens in Drosophila which are produced by genic interaction, a list which previously had included "antigen 1" and the lozenge antigen "L-3" (FOX 1949b; FOX and WHITE 1953; CHOVNICK and FOX 1953).

Some insight into the nature of the interaction between v^{1} and v^{36f}, and some further insight into the physiological and mutational properties of the two loci, is afforded by the observation that V-3 is present in Oregon R in what appears to be a haptenic form. If this is a true hapten a possible mechanism explaining its occurrence, as well as the presence of V-3 in v^{48a} and $v^{1}v^{36f}$, is the following: the wild alleles of both loci are necessary for the production of the V-3 hapten; v^{1} and v^{36f} involve complementary mutational changes in the sense that neither alone can support the production of the hapten but that both together can; these same complementary changes confer upon the two mutants the neomorphic property of participating together in bestowing upon the hapten complete antigenicity, possibly by coupling it with protein; v^{48a} involves a mutational change at its locus which does not prevent it from interacting with the wild allele at the other locus to produce the hapten, but which does confer upon it the neomorphic property of converting the hapten to complete antigen. The suggestion that genic action may involve the production of hapten-like prosthetic groups was first made some years ago by WRIGHT (1941) and has been applied more recently by EPHRUSSI (1950) to a case of cytoplasmic inheritance in yeast. The principal feature of the present suggestion is that the two vermilion loci appear to act as a single physiological unit in the production of V-3 specificity.

Returning to the problem outlined in the introduction, the demonstration that v^{48a} is transmitted as an allele of v^{1} but not of v^{36f} has immediate importance. Restated, the problem is created by the fundamental characteristic of pseudoallelism: pseudoallelic mutants are mutants which are transmitted as non-alleles since they exhibit a low order of recombination (crossing over), but which exhibit the physiological characteristics usually associated with allelism (similar or identical phenotypes in homozygotes and interaction in heterozygotes) (MCCLINTOCK 1944; LEWIS 1945). The usual interpretation given to such observations, and the one adopted here, is that such mutants occupy distinct but adjacent and duplicate loci on homologous chromosomes. The question is whether pseudoallelic loci are distinct physiologically and mutationally as well as in transmission. Further examination of this question as regards the vermilion mutants is facilitated by a tabulation of their physiological attributes in single mutant genotypes (table 7).

TABLE 7

Physiological attributes of vermilion mutants

Physiological attribute	v^1	v^{36f}	v^{48a}
Tryptophane accumulation	+	+	+
Response to kynurenine	+	+	+
Genetic suppression	+	−	−
Larval starvation	+	−	−
Antigen V-1	+	+	+
Antigen V-2	−	+	+
Antigen V-3	−	−	+

The features of this summary are such as to make apparent the complexity of the physiological relationships among these pseudoallelic mutants. Each mutant is characterized by its own constellation of physiological effects. The two mutants which are transmitted as alleles (v^1 and v^{48a}) show no greater physiological similarity than is exhibited by non-alleles. Indeed, v^{48a} shares more attributes with the non-allele v^{36f} than with its allele v^1. There is no single attribute nor any combination of attributes which distinguish the mutants at one locus from those at the other. In short, the two loci are not physiologically distinct.

Nor, is the situation less complex with respect to mutation. The suggestion that X-ray induced mutants always involve an alteration at the v^{36f} locus is contradicted by the allelic status of v^{48a}. Thus, the two loci are not separable on the basis of this aspect of mutability.

The centrality of v^{48a} in these considerations is, of course, recognized. It is possible that some of its special attributes, such as its association with antigen V-3, are a consequence of its origin by X-ray induction. There is, however, neither genetic nor cytological evidence that it is anything but a "point mutation" at the v^1 locus. On the other hand, it is possible that it is not an X-ray induced mutant even though it arose in an irradiated X chromosome. This would have implications for the argument concerning mutability but would not affect the physiological considerations.

The lack of physiological distinctiveness among pseudoallelic loci has been taken to require a revision of genetic theory (GOLDSCHMIDT 1951; PONTECORVO 1950; DEMEREC 1955); we prefer to suggest that the investigation of transmission, of physiology, and of mutation involves a different set of operations in each case, and that there is consequently no need to expect that the units which are thereby established will correspond (BRIDGMAN 1932; WRIGHT 1954). There is, in addition, difficulty in establishing an operational basis for the definition of a physiological unit. In the present case, for example, the whole pseudoallelic segment appears to function as a physiological unit at one level, but at a second level the individual loci exhibit physiological unity, while at a third level each locus appears to consist of two or more physiological units. It does not help, as has been suggested (LEWIS 1954, 1955), to propose that at the most fundamental level each of the genes of a pseudoallelic series acts as an agent controlling a single, specific reaction. In the present case again, there is disparity between the antigenic and pigmentary effects but there is no way of

knowing which most directly reflects "primary" gene action. In short, it is impossible to specify at the present time the operational criteria which would identify the primary level of gene action. When such criteria are available it will be possible to establish the unit of physiological action, and the question of its relationship to the unit of transmission may then be subjected to critical examination. Since similar difficulties exist in the definition of the unit of mutation, it would appear that the most satisfactory definition of the gene is still that of transmission, namely that of inseparability by crossing over and rearrangement.

SUMMARY

Immunogenetic analysis of the effects of three vermilion mutants has been performed by the use of coisogenic stocks. The X-ray induced mutant v^{48a} exhibits crossing over with the spontaneous mutant v^{36f}, but not with the spontaneous mutant v^{1}; the two latter are known to exhibit crossing over. Suppression tests with su^{o}-v reveal that v^{48a}, like v^{36f}, is unsuppressed, and larval starvation tests demonstrate an additional physiological similarity of v^{48a} and v^{36f}. In addition to residual antigens, which are present in all genotypes, three specific antigenic components have been identified: V-1, which is associated with v^{1}, v^{36f}, v^{48a}, and $v^{1}v^{36f}$; V-2, associated with v^{36f}, v^{48a}, and $v^{1}v^{36f}$; and V-3, associated with v^{48a} and $v^{1}v^{36f}$, and present in the wild genotype in apparently haptenic form. The "cis" and "trans" heterozygotes for v^{1} and v^{36f} possess antigens V-1 and V-2; there is no position effect on antigenic specificity. The implications of these results for the origin and physiology of pseudoallelism, and for gene-antigen relationships, are discussed. It is noted that while the pseudoallelic vermilion loci exhibit transmissional distinctness, there is neither physiological nor mutational distinctness. An operational interpretation is suggested.

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MAPPING CENTROMERES IN THE AXOLOTL[1]

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WHEN homologous loci separate from one another in the first meiotic division, the division is said to be reductional for those genes; if on the other hand sister loci separate, the division is said to be equational. Alternatively, these types of separation are termed prereductional and postreductional, respectively. According to most theories of meiosis, the centromere of each homologue remains undivided until the second maturation division; consequently, centromeres always separate prereductionally. Equational separation of markers can only be accomplished, therefore, if the original relation between marker and centromere is altered prior to completion of the first meiotic division. This is accomplished by exchange in the region between the marker and the centromere, the frequency of which is a function of the genetic length of this region. The relative proportions of reductional and equational first divisions, therefore, provide a measure of the distance from the marker in question to the centromere.

In organisms from which the products of meiosis are recovered singly it is not generally possible to determine whether the first division is equational or reductional. When half of or all the meiotic products are recoverable in a known order, as in Neurospora, equational and reductional separation of a marker can be scored and its location in relation to the centromere can be determined. Under special genetic conditions, centromere distance can be estimated from unordered tetrads (PERKINS 1949; WHITEHOUSE 1950; PAPAZIAN 1952). If one can contrive to recover routinely two strands from a single tetrad in one gamete, centromere location can be estimated in random gametes. This estimate is dependent on the knowledge of whether the strands carry sister or homologous centromeres or centromere regions. A case in point is the attached-X chromosome of *Drosophila melanogaster*. Caution must be exercised in concluding normal centromere distances from those measured in the special situations which allow recovery of two strands from a tetrad, since the special situation itself may alter exchange frequencies in the region under study. There is ample evidence of such altered frequencies in the case of the attached-X (BEADLE and EMERSON 1935). The distance from the locus of forked to the centromere as calculated from the rate of homozygosis of this marker in attached-X females is 5.0 units, whereas the distance from forked to bobbed, a region within but for all

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practical purposes identical in length with the region from forked to the centromere, is 9.2 units in free X females.

It is possible to recover more than a single strand from multivalents formed in polyploids and polysomics, and it might be guessed that an estimate of centromere distance could be obtained in these situations. Such an estimate is complicated, however, by altered recombination rates and irregular disjunction in multivalent associations.

A simple method is available for recovering a pair of chromatids from each tetrad in the ova of diploid axolotls and other amphibians. Oogenesis in the axolotl is arrested at metaphase of the second division until fertilization, which is effected in the cloaca of the females; the eggs are laid immediately following fertilization, and the second maturation division is completed within one hour thereafter. Refrigeration of freshly laid eggs often brings about the complete inhibition of this division, giving rise to a diploid egg and subsequently a triploid zygote (FANKHAUSER and HUMPHREY 1942). Since the two strands so recovered from each tetrad segregated together at anaphase 1, they presumably contain sister centromeres. Furthermore, the fact that the first meiotic division of a normal diploid oocyte is completed and the first polar body is separated from the oocyte prior to refrigeration eliminates as complications in this case the possibility that recombination frequencies are altered by the treatment or that any material from the first polar body is included in the egg nucleus.

Consider the eggs of a female axolotl of the constitution A/a, in which the second maturation division is inhibited. In the absence of exchange between the *a* locus and the centromere, separation is reductional and the products of the first meiotic division are *AA* and *aa* in equal numbers; following a single exchange between *a* and the centromere, separation is equational, and both products of the first meiotic division are of the constitution *Aa*. Fertilization of these diploid eggs by *a*-bearing sperm yields triploids of constitution *AAa*, *aaa*, and *Aaa*, respectively. It can be seen that one minus two times the frequency of the homozygous recessive class equals the frequency of equational separation of *a*.

For the present treatment, a tetrad will be defined to consist exclusively of the region between a marker and its centromere; it has been shown in the preceding paragraph that no-exchange and single-exchange tetrads yield 100% reductional separation and 100% equational separation, respectively. Tetrads of higher rank, however, yield both types in varying proportions, depending on rank. The following equation of MATHER (1935) expresses equational separation as a function of rank:

$$E_r = \tfrac{2}{3}[1 - (-\tfrac{1}{2})^r]$$

It can be seen that as r increases, E_r rapidly approaches 0.66. From equations of RIZET and ENGELMANN (1949) one can express the frequency of equational separation as a function of $\bar{r}$, the mean rank:

$$E_{\bar{r}} = \tfrac{2}{3}(1 - e^{-\frac{3}{2}\bar{r}})$$

$\bar{r}$ is in turn a function of the map distance in that the mean rank is twice the map distance, D, expressed in Morgans (M), e.g., an average of one exchange per tetrad corresponds to 50% recombination or 0.5 M. Consequently, $2D$ can be substituted

for $\bar{r}$ in the above equation to express the frequency of equational separation directly as a function of centromere distance. This expression is idealized in that it assumes independence of successive exchanges, i.e., that the frequency of tetrads with no, one, two, etc., exchanges follows a Poisson distribution.

It should be possible, by these relations, to determine centromere distances of the known genes in the axolotl from the frequency of recessives among the induced triploid progeny of heterozygous females by homozygous recessive males.

MATERIALS AND METHODS

The characters utilized in this investigation are (1) sex, (2) body color as influenced by a recessive gene, *d*, which inhibits melanoblast migration (DALTON 1950) resulting in white animals, and (3) development as influenced by a recessive gene, *f*, which causes a marked fluid imbalance during early embryonic development (see HUMPHREY 1948, 1952). All three loci are unlinked, and classification of homozygous recessives for each locus is unambiguous.

Female heterogamety (ZW) and male homogamety (ZZ) were demonstrated in the Mexican axolotl by HUMPHREY (1945). Although Z refers to the chromosome which carries the factor or factors that determine maleness, for present purposes it is convenient to think of it as being nothing more than the recessive allele of W, which can be considered as a single factor determining femaleness.

The sex of both diploid and triploid axolotls was determined in many instances by examination of the gonads of adults. In animals sacrificed in larval stages, the gonads were examined under low-power magnification at autopsy; any not then identified with certainty were studied in serial sections.

Induction of triploidy was accomplished by refrigeration of freshly laid eggs in the manner previously described by FANKHAUSER and HUMPHREY (1942). As a rule, the period of refrigeration was four to eight hours, and the temperature was maintained between 0 and 3 degrees centigrade; following this treatment the eggs were placed in water at room temperature. Larvae not requiring rearing were fixed entire in Bouin's fluid shortly after hatching. When it was necessary to rear the larva, as for identification of its sex, a part of its tail was removed shortly after hatching and similarly fixed in Bouin's. All tailtips were stained in Harris' acid hemalum and mounted entire. Chromosome number was determined from counts on mitotic figures in the epidermis of the transparent tail fin, or by study of nuclear size and number of nucleoli in interphase nuclei in those preparations from which counts were unobtainable.

RESULTS

The results of the experiments described are summarized in tables 1 to 3. These tables show the distribution of phenotypes among the treated and untreated progeny of WZ, *Dd*, and *Ff* females respectively when crossed with homozygous recessive males. The sum of the chi squares of the six diploid samples calculated on an expectation of a 1:1 ratio is 4.74, corresponding to a probability of 0.45; thus there is no significant deviation from the expected 1:1 ratio among the diploid progeny. Among the triploid progeny of WZ females, the proportion of males is 0.156; this figure

corresponds to 0.688 equational separation of the sex locus. The frequency of homozygosis for *d* is 0.153, corresponding to 0.694 equational separation. The previously observed absence of linkage between sex and *d* is also observed in these crosses; among the triploids which were fixed at a stage in which sex could be determined, the sex ratios found in the white (26♀ : 4♂) and the dark animals (90♀ : 17♂) did not differ. The frequency of homozygosis for *f* is 0.138, corresponding to 0.724 equational separation.

DISCUSSION

The observed frequency of equational separation, *E*, for each of the three genes studied in the axolotl is approximately 0.66. It is evident from the equations presented in the introductory portion of this paper that the observed values of *E* agree very well with the theoretical limiting value of *E* which is approached as the number of exchanges, and thus the map distances between the gene and the centromere becomes large. It may be concluded, therefore, that the three loci utilized in the present study are located toward the ends of chromosome arms. GALL (personal communication) has observed in lampbrush chromosome bivalents dissected from oocytes of *Ambystoma tigrinum*, a species closely related to the Mexican axolotl, that the mean distance between chiasmata tends to be constant regardless of chromosome length. This internodal distance is such that the shortest chromosome, ca. 270 μ, averages three chiasmata per bivalent (five bivalents observed) and the four longest chromosomes, 900–1000 μ, average 7.6 chiasmata per bivalent (five bivalents observed). In a triploid axolotl, he also observed up to eight or nine

TABLE 1

Sex of triploid and diploid progeny from WZ ♀ × ZZ ♂ matings

Spawning	Treated eggs				Untreated eggs	
	Triploid		Diploid		Diploid	
	WWZ or WZZ ♀♀	ZZZ ♂♂	WZ ♀♀	ZZ ♂♂	WZ ♀♀	ZZ ♂♂
65	19	7	2	0	5	7
69	5	3	5	4	7	5
99	6	3	—	—	—	—
103	20	0	7	6	2	4
106	2	0	7	6	20	26
108	8	0	4	1	27	30
109	4	0	3	3	27	19
635	72	15	1	—	8	8
671	11	1	—	2	—	—
676	33	5	3	1	21	10
682	20	3	—	—	—	—
Total	200	37	32	23	117	109
Frequency of males	0.156		0.418		0.482	

TABLE 2

Color of triploid and diploid progeny from Dd ♀ × dd ♂ *matings*

Spawning	Treated eggs				Untreated eggs	
	Triploid		Diploid		Diploid	
	DDd or *Ddd* dark	*ddd* white	*Dd* dark	*dd* white	*Dd* dark	*dd* white
103	20	3	13	11	113	137
635	118	25	12	18	117	128
671	14	1	16	17	100	84
676	44	8	17	14	114	108
719	8	0	19	12	92	53
Total	204	37	77	72	536	510
Frequency of white	0.153		0.483		0.487	

TABLE 3

Phenotype of triploid and diploid progeny from Ff ♀ × ff ♂ *matings*

Spawning	Treated eggs				Untreated eggs	
	Triploid		Diploid		Diploid	
	FFf or *Fff* normal	*fff* lethal	*Ff* normal	*ff* lethal	*Ff* normal	*ff* lethal
692	46	9	11	7	95	84
725	12	3	37	22	22	30
738	47	7	10	5	—	—
763	120	17	15	22	65	55
Total	225	36	73	56	182	169
Frequency of lethal	0.138		0.434		0.482	

chiasmata per bivalent (trivalents were also observed). If the chiasmata described by GALL (1954) for lampbrush chromosomes are actually correlated with exchange, as he suspects, the conclusion that the three genes studied are considerably remote from the centromere is quite plausible. It is noteworthy that this conclusion adds support to the idea that the sex-determining region of the sex chromosome is relatively limited and may constitute a single locus, rather than a large proportion of the chromosome.

Prereduction of centromeres at metaphase 1 is an implicit assumption in most considerations of the genetic consequences of meiosis, although there are few organisms in which there is genetic evidence in favor of such a contention. In this context, the presently observed values of E are interpreted as recovery of sister centromeres accompanied by recovery of a random pair of loci. It should be pointed

out, however, that random pairwise separation of centromeres from the bivalent would result in the recovery of a random pair of loci regardless of the position of the gene on the chromosome. The simplest way to rule out such behavior as the explanation of the current observations is to find a gene that separates equationally with some frequency less than 0.66; only three genes are presently available for testing in the axolotl, however. It might be considered that the sex locus of any amphibian in which triploidy can be induced as described and in which the female is heterogametic might provide evidence on the point in question. It is known that *Triturus viridescens* fulfills the former of these requirements (FRANKHAUSER and GRIFFITHS 1939), and there is reason to suspect that it also fulfills the latter; unfortunately, however, diploid ratios in laboratory populations of this species are so variable that sex ratios of triploids would not be a reliable index of equational separation. At present, therefore, it is not possible to distinguish between random first meiotic separation of chromatids at a particular locus caused by frequent exchange between the locus and the centromere and that caused by random separation of centromeres from the bivalent.

SUMMARY

By refrigeration of freshly laid eggs of the Mexican axolotl (*Ambystoma mexicanum*), it is possible to inhibit the second meiotic division to produce diploid ova. Reductional separation of *A* from *a* in a female of constitution *A/a* yields diploid ova of constitutions *AA* and *aa* in equal numbers; equational separation yields *Aa* ova. Fertilization of these ova with *a*-bearing sperm results in triploid zygotes of constitutions *AAa*, *aaa*, and *Aaa*, respectively. The frequency of equational separation of three different markers determined from the proportion of homozygous recessive triploids among the refrigerated progeny of heterozygous females by homozygous recessive males was approximately 0.66. On the basis of the known relation existing between the frequency of equational separation and the map distance between the centromere and a linked marker, it is concluded that the three genes studied are located toward the ends of chromosome arms. An alternative interpretation of the data is that in the axolotl centromeres separate in random pairs from the bivalent at the first maturation division.

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A GENERALISED ANALYSIS OF DIALLEL CROSSES*

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BASED on the methods of MATHER (1949) a technique of analysing diallel crosses between homozygous parents has recently been developed by JINKS and HAYMAN (1953; HAYMAN 1954; JINKS 1954). They are able to estimate the genetic parameters, D_1, H_1, H_2 and F (following MATHER's notation) and their standard errors from second degree statistics, and, thence, to estimate such genetic components as dominance and allele frequency. The present paper details the extension of these methods of diallel analysis to crosses involving parental heterozygosity. The required experimental design comprises all possible crosses, including reciprocals and selfing, among a number of individuals, and the proposed analysis estimates both the degree of parental heterozygosity and comparable genetic components of variation to those of the homozygous analysis.

Before embarking on a full description of our method of analysis, a preliminary discussion of notation is necessary. In order to specify completely genotype frequencies in a population with three genotypes, two independent parameters are needed. Many schemes involving two such parameters are, no doubt, possible; two schemes will be detailed and a discussion of their relative merits will be useful. Thus, in terms of a one-gene, two-allele model, it is possible to specify the unknown parental population in terms of the parameters u and v (i.e. $v = 1 - u$), the allele frequencies, and f, the coefficient of inbreeding.

Thus,

Genotype	*Frequency*
AA	$u^2 + uvf$
Aa	$2uv(1 - f)$
aa	$v^2 + uvf$

Alternatively, the parental population can be specified as—

Genotype	*Frequency*	
AA	α	where $\alpha + \beta + \gamma = 1$
Aa	β	
aa	γ	

The $\alpha\beta\gamma$ system has been chosen to illustrate the development of the method of diallel analysis in preference to the uvf scheme, even though the latter has the advantage of being in line with current longstanding notation (e.g. WRIGHT 1923; LERNER 1950). At important stages, however, both uvf and $\alpha\beta\gamma$ forms will be given. The two systems are, obviously, completely interchangeable, differing only in the form of presentation of the fundamental genetic variables. Thus, one feature of the $\alpha\beta\gamma$ system is the more obvious manner in which heterozygosity is specified as

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TABLE 1

Genotype / Frequency / Parents / Mean			*AA* α $+d$	*Aa* β h	*aa* γ $-d$
AA	α	$+d$	*AA* α^2 $+d$	*AA*:*Aa* $\alpha\beta$ $\frac{1}{2}(d + h)$	*Aa* $\alpha\gamma$ h
Aa	β	h	*AA*:*Aa* $\alpha\beta$ $\frac{1}{2}(d + h)$	*AA*:2*Aa*:*aa* β^2 $\frac{1}{2}h$	*Aa*:*aa* $\beta\gamma$ $\frac{1}{2}(-d + h)$
aa	γ	$-d$	*Aa* $\alpha\gamma$ h	*Aa*:*aa* $\beta\gamma$ $\frac{1}{2}(-d + h)$	*aa* γ^2 $-d$
Array mean			$\alpha[(\alpha + \frac{1}{2}\beta)d + (\gamma + \frac{1}{2}\beta)h]$	$\beta[(\alpha - \gamma)\frac{1}{2}d + \frac{1}{2}h]$	$\gamma[(\gamma + \frac{1}{2}\beta)(-d) + (\alpha + \frac{1}{2}\beta)h]$

Overall F_1 mean $= (\alpha - \gamma)d + (2\alpha\gamma + \beta - \frac{1}{2}\beta^2)h$

compared with the alternative notation where the heterozygosity appears in a "relative" form as positive or negative inbreeding. An important aspect of the chosen notation is that, by specifying the three genotypes separately, the expected statistics are presented in a form in which the effect of selection on the phenotype can be more readily observed. An example of this aspect is given by LERNER (1954) involving models directly comparable with the present $\alpha\beta\gamma$ notation, in which different selective values are assigned to the several genotypes. A comparable instance is given by HAYMAN and MATHER (1953) where the consequences are investigated of the selective advantage of heterozygotes.

In terms of a one-gene model, the notation of MATHER (l.c.) is used for the description of mean values; namely, the mid-homozygous value is zero and "*A*" adds on $+d$ and "*a*" $-d$, while the heterozygote deviates by h from the mid-homozygote. In terms of this gene, the contributions to the offspring family means and their frequencies, in the diallel cross, are shown in table 1.

Table 1 is used to calculate expected variances for parents, arrays, array means etc. and the covariances for array family-means with non-recurrent parents. (The term "array" refers to the family-means of all the offspring of one parental line in the diallel cross.)

For instance, the average variance ($\bar{V}_r$) of family-means around the array mean is,

$$\alpha(V_{AA\text{ array}}) + \beta(V_{Aa\text{ array}}) + \gamma(V_{aa\text{ array}})$$

Substituting the actual variances this becomes

$$\begin{aligned}\bar{V}_r &= \alpha\{\alpha d^2 + \tfrac{1}{4}\beta(d + h)^2 + \gamma h^2 - [(\alpha + \tfrac{1}{2}\beta)d + (\gamma + \tfrac{1}{2}\beta)h]^2\} \\ &\quad + \beta\{\tfrac{1}{4}\alpha(d + h)^2 + \tfrac{1}{4}\beta h^2 + \tfrac{1}{4}\gamma(-d + h)^2 - [(\alpha - \gamma)\tfrac{1}{2}d + \tfrac{1}{2}h]^2\} \\ &\quad + \gamma\{\alpha h^2 + \tfrac{1}{4}\beta(-d + h)^2 + \gamma d^2 - [(\gamma + \tfrac{1}{2}\beta)(-d) + (\alpha + \tfrac{1}{2}\beta)h]^2\} \\ &= \alpha\{[\tfrac{1}{4}\beta(1 - \beta) + \alpha\gamma]d^2 + [\tfrac{1}{4}\beta(1 - \beta) + \alpha\gamma]h^2 - [\tfrac{1}{2}\beta(1 - \beta) + 2\alpha\gamma]dh\} \\ &\quad + \beta\{[\tfrac{1}{4}\beta(1 - \beta) + \alpha\gamma]d^2\} \\ &\quad + \gamma\{[\tfrac{1}{4}\beta(1 - \beta) + \alpha\gamma]d^2 + [\tfrac{1}{4}\beta(1 - \beta) + \alpha\gamma]h^2 + [\tfrac{1}{2}\beta(1 - \beta) + 2\alpha\gamma]dh\}\end{aligned}$$

If Z is defined as $\frac{1}{4}\beta(1-\beta)+\alpha\gamma$,

$$\bar{V}_r = Z[d^2+(\alpha+\gamma)h^2-2(\alpha-\gamma)dh]$$

For a large number of genes which are randomly distributed in the parents and which show no interaction.

$$\bar{V}_r = \Sigma Z[d^2+(\alpha+\gamma)h^2-2(\alpha-\gamma)dh]$$

In the homozygous diallel the offspring on the leading diagonal of the diallel table are identical with the parents; this is not so, however, in the present case. The offspring of the heterozygous × heterozygous cross of the one-gene model, show a fall in mean equal to $\frac{1}{2}h$, from the parental value. It is thus possible to calculate, both the variance of the actual parents (V_{P1}) and also that of the offspring family means on the leading diagonal, which can be regarded as a fictitious parental population (V_{P2}). They are found to be

$$V_{P1} = 4Zd^2+\beta(1-\beta)h^2-2\beta(\alpha-\gamma)dh$$

$$V_{P2} = 4Zd^2+\tfrac{1}{4}\beta(1-\beta)h^2-\beta(\alpha-\gamma)dh$$

A variety of other statistics can be calculated in a comparable manner and, in terms of these various statistics, the population can be described using the genetic components of variation listed below.

Parameter	*'αβγ' system*	*'uvf' system*
D_I	$8\Sigma Zd^2$	$4\Sigma uv(1+f)d^2$
D_{II}	$2\Sigma\beta d^2$	$4\Sigma uv(1-f)d^2$
H_I	$16\Sigma(\alpha+\gamma)Zh^2$	$8\Sigma uv(1+f)(2uvf-2uv+1)h^2$
H_{II}	$64\Sigma Z^2h^2$	$16\Sigma u^2v^2(1+f)^2h^2$
H_{III}	$4\Sigma\beta(\alpha+\gamma)h^2$	$8\Sigma uv(1-f)(2uvf-2uv+1)h^2$
H_{IV}	$16\Sigma\beta Zh^2$	$16\Sigma u^2v^2(1-f)(1+f)h^2$
F_I	$32\Sigma(\alpha-\gamma)Zdh$	$16\Sigma uv(1+f)(u-v)dh$
F_{II}	$8\Sigma\beta(\alpha-\gamma)dh$	$16\Sigma uv(1-f)(u-v)dh$

Note that F_I and F_{II} can be either both positive or both negative; all other components of variation are positive. Substituting the above components of variation, the variances and covariances become

$$\begin{aligned}
V_{P1} &= \tfrac{1}{2}D_I+\tfrac{1}{4}H_{III}-\tfrac{1}{4}F_{II}\\
V_{P2} &= \tfrac{1}{2}D_I+\tfrac{1}{16}H_{III}-\tfrac{1}{8}F_{II}\\
\bar{V}_r &= \tfrac{1}{8}D_I+\tfrac{1}{16}H_I-\tfrac{1}{16}F_I\\
V_{\bar{r}} &= \tfrac{1}{8}D_I+\tfrac{1}{16}H_I-\tfrac{1}{16}H_{II}-\tfrac{1}{16}F_I\\
\bar{W}_{P1/r} &= \tfrac{1}{4}D_I+\tfrac{1}{8}H_{III}-\tfrac{1}{8}H_{IV}-\tfrac{1}{16}F_I-\tfrac{1}{16}F_{II}\\
\bar{W}_{P2/r} &= \tfrac{1}{4}D_I+\tfrac{1}{16}H_{III}-\tfrac{1}{16}H_{IV}-\tfrac{1}{16}F_I-\tfrac{1}{32}F_{II}\\
W_{P1/P2} &= \tfrac{1}{2}D_I+\tfrac{1}{8}H_{III}-\tfrac{3}{16}F_{II}
\end{aligned}$$

Where $\bar{V}_r$ is mean variance of arrays, $V_{\bar{r}}$ is variance of array means, $\bar{W}_{P1/r}$ and $\bar{W}_{P2/r}$ are the mean covariances of arrays with $P1$ and $P2$ respectively and $W_{P1/P2}$ is the covariance of $P1$ and $P2$.

These formulae may be rearranged as follows:

$$
\begin{aligned}
D_I &= 2V_{P1} + 8V_{P2} - 8W_{P1/P2} \\
H_I &= 4V_{P1} + 16V_{P2} + 16\bar{V}_r + 16\bar{W}_{P1/r} - 32\bar{W}_{P2/r} - 16W_{P1/P2} \\
H_{II} &= 16\bar{V}_r - 16V_{\bar{r}} \\
H_{III} &= 16V_{P1} + 16V_{P2} - 32W_{P1/P2} \\
H_{IV} &= 8V_{P1} - 16\bar{W}_{P1/r} + 16\bar{W}_{P2/r} - 8W_{P1/P2} \\
F_I &= 8V_{P1} + 32V_{P2} + 16\bar{W}_{P1/r} - 32\bar{W}_{P2/r} - 32W_{P1/P2} \\
F_{II} &= 16V_{P1} + 32V_{P2} - 48W_{P1/P2}
\end{aligned}
$$

The components of variation have been defined such that in a random mating population, they reduce to those of MATHER (l.c.). The only difference is that MATHER's D contains a small h component if there is inequality of allele frequency. This component is herein represented separately as F_I, F_{II}, H_I and H_{III} following the modification used by JINKS and HAYMAN (1953).

Thus under random mating—

$$
\begin{aligned}
D_I &= D_{II} = 4\Sigma uvd^2 \\
H_I &= H_{III} = 8\Sigma uv(1 - 2uv)h^2 \\
H_{II} &= H_{IV} = 16\Sigma u^2v^2h^2 \\
F_I &= F_{II} = 16\Sigma uv(u - v)dh
\end{aligned}
$$

For random mating with unequal allele frequencies, F_I and F_{II} are only zero under special conditions of opposing d's and h's.

Other properties of the components of variation are:

(i) for equal allele frequencies, irrespective of the mating system,

$$H_I = H_{II}, H_{III} = H_{IV} \text{ and } F_I = F_{II} = 0$$

(ii) therefore, for random mating and equal allele frequencies,

$$H_I = H_{II} = H_{III} = H_{IV} \text{ and } F_I = F_{II} = 0.$$

(iii) under all mating systems, irrespective of allele frequencies,

$$\frac{H_I}{H_{III}} = \frac{H_{III}}{H_{IV}}$$

(iv) for a completely inbred population,

$$F_{II} = H_{III} = H_{IV} = 0$$

and the other components of variation become those for a diallel between inbred lines, namely, D, H_1, H_2 and F (JINKS 1954).

The estimates of variance and covariance given above must be corrected for variance due to non-heritable sources, prior to computation of the genetic components of variation. Thus, each estimated variance includes the non-heritable variance of family-means, comparable with the E_2 component used by MATHER (l.c.). In addition, the covariance estimates require correction for a non-heritable component, details of which are fully discussed by JINKS (1954). The covariance correction in question is $1/n$th of E_2 where n is the number of parental lines in the diallel cross. E_2 is calculated from the original data as the error variance of family-means, for instance from differences between replicates.

Following these corrections for non-heritable variance, the genetic components of variation can be estimated as above. The genetic constitution of the population under investigation is then deducible in the following manner.

Dominance

The expression

$$\sqrt{\frac{\frac{1}{2}(H_I + H_{IV})}{D_I}}$$

gives a weighted estimate of the mean level of dominance. This reduces to

$$\sqrt{\frac{\sum Z_i h_i^2}{\sum Z_i d_i^2}}$$

which becomes h/d if the n loci have equal effects; whereas if the loci have unequal effects, the estimated dominance ratio is automatically weighted in favour of those with largest effect, with allele frequency nearest to 0.5, and with least heterozygosity among the parents. As unequal effects will be the rule rather than the exception, and since we can only deal with the combined effects of all the loci, the weighted estimate will include relatively more information about loci of greater consequence in these crosses than about loci at low frequency and/or of small effect.

In addition to the above, on similar grounds

$$\sqrt{\frac{H_{II}}{D_I^2}} = \sqrt{\frac{64\sum Z_i^2 h_i^2}{64\sum Z_i^2 d_i^4}} = \frac{h}{d^2}$$

The estimates of h/d and h/d^2 can be solved simultaneously for h and d.

Average level of heterozygosity (β)

Of the estimates of this parameter which are available, some depend on comparisons within H's, one on comparisons within F's and another on comparisons within D's. Estimates depending on H's will refer to the average level of heterozygosity at loci showing dominance since the ratios are restricted to terms in h^2 and they are weighted in favour of loci showing greater dominance since such loci contribute relatively more than ones showing little dominance. The estimate based on F's again involves only loci showing dominance, but weighting in favour of loci showing greater dominance is less than in estimates based on H's. The remaining estimate, that using D's, takes into account all loci, provided that they show no linkage. This latter restriction is serious but necessary because D_{II}, as shown later herein, is derived from within family variance.

The estimates of β are

$$\beta \doteqdot \frac{H_{IV}}{H_I + H_{IV}} \doteqdot \frac{H_{II}\, H_{III}}{H_{II}\, H_{III} + H_I^2}$$

$$\frac{\beta}{Z} \doteqdot \frac{4F_{II}}{F_I} \doteqdot \frac{4D_{II}}{D_I}$$

The two estimates using H's will be weighted in favour of loci with allele frequency nearest to 0.5. In addition, loci with a low level of heterozygosity contribute relatively more to these estimates than ones with a high level; hence underestimation of the mean heterozygosity will result whenever β has a different value for different loci, which will in practice be the normal situation.

The three ratios

$$\frac{4H_I}{H_{II}} \doteqdot \frac{4H_{III}}{H_{IV}} \doteqdot \frac{(1-\beta)}{Z} \text{ and } \frac{4H_{III}}{H_I} \doteqdot \frac{\beta}{Z}$$

are useful in giving the relative values of β and Z. They are not, however, independent of the previous estimates involving H's and must therefore only be used as alternative, but not additional, estimates.

By solving the above equations for both β and Z, it is also possible to estimate the mean value of $\alpha\gamma$, since

$$Z = \alpha\gamma + \tfrac{1}{4}\beta(1-\beta)$$

Inbreeding coefficient of parental population (f)

In terms of the $\alpha\beta\gamma$ notation, the inbreeding coefficient is given by

$$f = \frac{4\alpha\gamma - \beta^2}{(2\alpha+\beta)(\beta+2\gamma)}$$

whence

$$f \doteqdot \frac{H_I - H_{III}}{H_I + H_{III}} \doteqdot \frac{H_{II} - H_{IV}}{H_{II} + H_{IV}} \doteqdot \frac{F_I - F_{II}}{F_I + F_{II}} \doteqdot \frac{D_I - D_{II}}{D_I + D_{II}}$$

These estimates of f are weighted in a manner comparable with the estimates of β.

Interpretation of the sign of the F_I and F_{II}

The sign is dependent on the composite sign of $(\alpha - \gamma)h$, both components of which can take either sign.
Thus,

Sign of $(\alpha - \gamma)$	*Sign of* h	*Therefore, when there is an excess of*	*The sign of both* F_1 *and* F_{II} *will be*
−	+	dominant increasers	−
−	−	recessive increasers	+
+	+	recessive decreasers	+
+	−	dominant decreasers	−

Consequently, when F_I and F_{II} are positive there is an excess of dominants and, when negative, recessives are in excess.

For completeness, it is worthwhile noting at this point that the difference, $2\overline{P}_2 - \overline{P}_1$, will indicate whether there is a preponderance of increasers or of decreasers. Since

$$2\overline{P}_2 - \overline{P}_1 = \Sigma(\alpha - \gamma)d$$

a positive result will indicate an excess of increasers while the result will be negative when decreasers are in excess.

Number of loci showing dominance

An estimate of this is given by the expression

$$\frac{16(\bar{F}_1 - \bar{P}_2)^2}{H_{II}} = \frac{(2\Sigma Zh)^2}{4\Sigma Z^2h^2}$$

provided that certain assumptions are made. Thus, assuming that the n loci involved have equal effects and show unidirectional dominance, the expression becomes

$$\frac{(2nZH)^2}{4nZ^2h^2} = \frac{n^2}{n} = n$$

This will be an underestimate of n if either or both assumptions are incorrect. Thus, when all loci do not show unidirectional dominance, + and − values of h cancel out in F_1 and P_2 means, with a consequent reduction in the estimated value of n. In addition, inequality of effects at the n loci will also give a spuriously low estimate, the reasons for which, in a comparable estimate, are discussed by MATHER (l.c.).

It is obvious that the present estimate, being restricted to a comparison of terms in h, will give an estimate for loci showing dominance and will be biased in favour of those of greater dominance. No similar comparison involving d's has been found, hence there is no available estimate of MATHER's 'effective factors'.

Linkage

The generalised diallel analysis, as described above, has been restricted to the analysis of family-means, and at the F_1 level, the use of family-means avoids the consequences of linkage. If, however, individuals are used instead of family-means, linkage phenomena and their resulting complications become involved.

Calculations of the total variance of *individuals* in a diallel is possible in a manner comparable to that for means. This variance will include an environmental component different from that for variance of means, in this case being the one for individuals, E_1 (MATHER, l.c.).

The variance of individuals about the grand mean (V_o) is,

$$\tfrac{1}{4}D_I + \tfrac{1}{4}D_{II} + \tfrac{1}{8}H_I - \tfrac{1}{16}H_{III} + \tfrac{1}{4}\Sigma\beta h^2 - \tfrac{1}{8}F_I - \tfrac{1}{8}F_{II}$$

where D_{II} (as defined on page 67) is $2\Sigma\beta d^2$. This variance is computed on the assumption of no linkage. If linkage is an important element, the actual variance will deviate from this expectation. It has not been possible to specify whether preponderant coupling or repulsion linkage will increase or decrease the actual variance. This difficulty arises from the joint effect of the frequencies of the various parental genotypes and the particular linkage conditions in each parent.

Theoretically, it should be possible to detect linkage if the several H's, F's and D_I (which have been computed from the family-means) are substituted in the above formula for V_o—this will only be possible, however, if no other source of bias is present, such as unequal effects. The item ($\frac{1}{2}\Sigma\beta d^2 + \frac{1}{4}\Sigma\beta h^2$) can then be de-

termined both by remainder and also by substitution of β, d^2 and h^2 which have been estimated. The value of this item should be the same by remainder as by substitution if linkage is absent, if allele frequencies are uncorrelated and if all loci show dominance and have equivalent effects. Any difference between "remainder" and "substitution" values will indicate the general level of bias from the combined sources or, in the special case of equal effects and uncorrelated allele frequencies, linkage will be indicated.

The accuracy of the method outlined above will not, however, be very great, since the components of variation will have relatively large standard errors. No significance tests have, however, been devised.

In a diallel between homozygous lines the two new items $\frac{1}{2}\Sigma\beta d^2 + \frac{1}{4}\Sigma\beta h^2$ are both equal to zero. The linkage test, therefore, is simply one of homogeneity of the remaining components of variation derived from the analysis of family means and those from the family variances of segregating families. Since segregating families do not appear in the parents and first generation progeny of a homozygous diallel this test cannot be applied until at least segregating generations such as F_2's have been raised.

Non-allelic interaction

In all the statistics which have been developed non-allelic interaction will appear as mimicking allelic interaction (i.e. dominance, $h \leq d$ or overdominance $h > d$). Thus, for instance, duplicate factor interaction will appear as $h \leq d$, whilst complementary gene action will appear as $h > d$. Other aspects of non-allelic interaction are considered in the section immediately following.

The regression of array covariance (W_r) on array variance (V_r)

A brief summary of the conclusions of JINKS (1954) and HAYMAN (1954) relating to the homozygous analysis will form a useful basis for discussion of the present general case. Thus, with parental homozygosity, and in the absence of non-allelic interaction, the regression of W_r on V_r represents a line of unit slope for any particular level of dominance, whilst in the absence of dominance the regression line degenerates to a single point (see fig. 1). All points must, on mathematical grounds, be within a limiting parabola, as indicated in figure 1. The point of intersection of the regression line with the covariance axis, relative to the position of $\frac{1}{4}V_{P1}$ on that axis, can be used to estimate the overall degree of dominance. The order of parents along the regression line indicates the relative proportion of dominants to recessives in the corresponding parents—points with lower values of W_r and V_r have the greater proportion of dominants. Non-allelic interaction, particularly complementary gene action, results in deviation of the points corresponding to the arrays of interacting parents from the expected regression line. Hence, in such cases, the actual regression line will usually deviate from unit slope.

Returning to our main theme, in the generalised case, a two-gene, two-allele model can usefully illustrate the consequences of heterozygosity of one or more of the parents. The nine parents in the diallel model show all combinations of increasing and decreasing alleles of two genes, A and B. For simplicity, both genes are shown

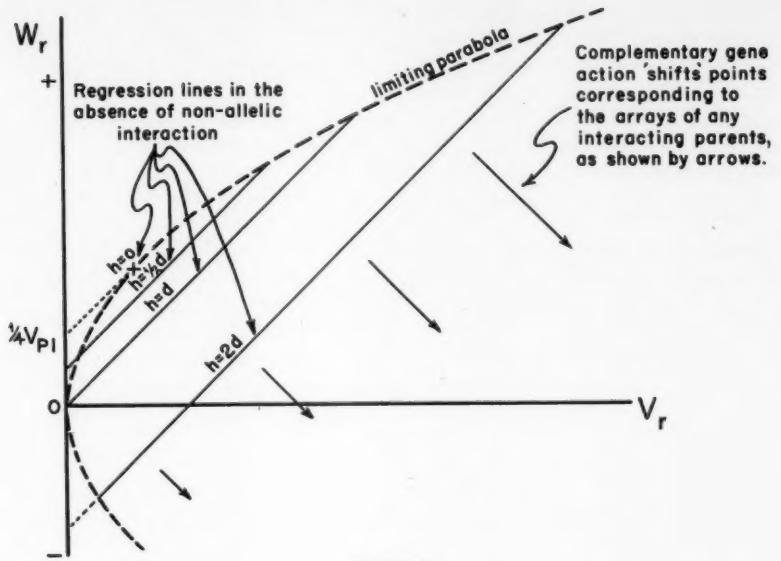

FIGURE 1.

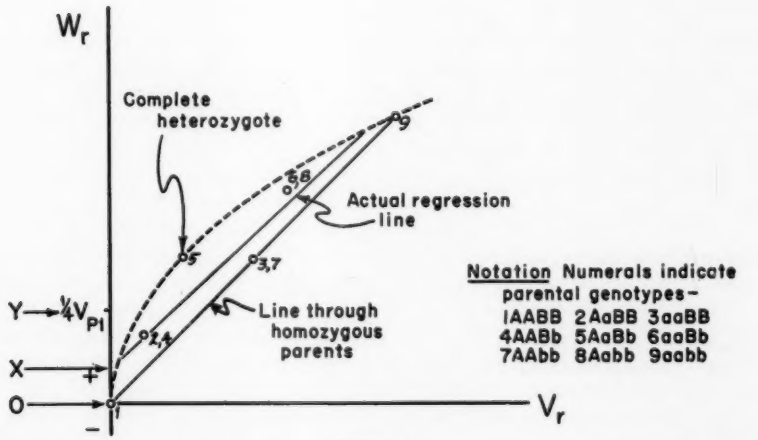

FIGURE 2.

with equal effects and interaction between loci is excluded. Family means are used in the calculation of array variances and covariances. In the graphs (fig. 2 and 3) *A* and *B* represent dominant increasing alleles and *a* and *b* represent recessive decreasing alleles, for the various levels of h/d.

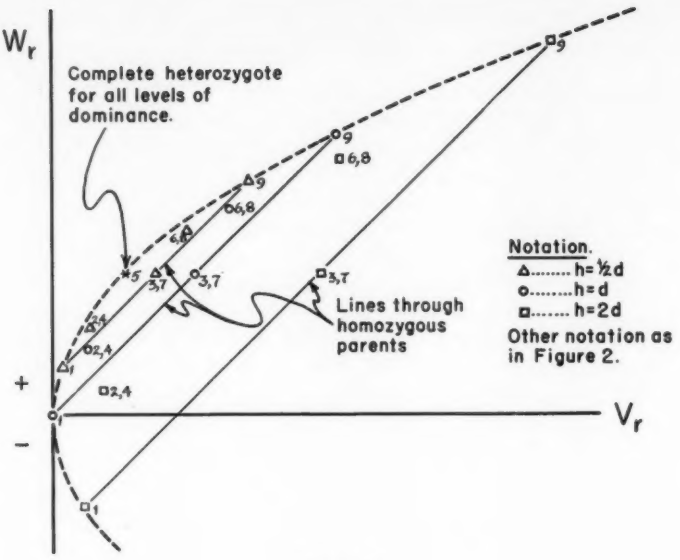

FIGURE 3.

In figure 2, the W_r/V_r graph is shown of the model for full dominance. Points representing homozygous parents are exactly as in a homozygous-only diallel; thus a line through such points is of unit slope. The complete heterozygote (*AaBb*) lies on the limiting parabola at a point which, in a homozygous diallel, represents that for parents showing no dominance. The points for partial heterozygotes are interspersed between that for the complete heterozygote, on the one hand, and, on the other hand, those for homozygous parents. The actual regression line for the points in figure 2 is illustrated; its slope deviates from unity ($b = .92 \pm .01$) even though the model excludes non-allelic interaction. In an actual cross, the regression line will only rarely be of unit slope, the actual slope of the line will depend on the heterozygosity of the parents present (excluding interaction). The order of points along the regression line indicates the relative proportions of dominants to recessives in each parent, just as with the homozygous analysis. In practice, however, this will be more difficult to determine in the generalised case due to the scatter of points about the regression line.

Figure 3 is similar to figure 2 but includes various levels of dominance: it will be evident that points for heterozygous parents always lie to the left of and above those for homozygous parents. With an increasing proportion of heterozygosity in the parents, for any particular level of dominance, there is a proportional shifting of the regression line upwards and to the left. The effect of this shift is to simulate lower levels of dominance.

Except in cases of extreme over-dominance, the points for heterozygous parents

lie in a relatively restricted area, the long-axis of which is roughly parallel to a line of unit slope. Therefore, the deviation of the regression line from unit slope due to heterozygosity will not usually be very great, though the deviation may be significant. As mentioned above, it has been shown that complementary gene action in particular, can cause extreme deviation of the corresponding point to the right of and below the expected regression line. Hence it is considered that any interaction causing extreme deviation, will not be confused with heterozygosity using this regression method, but confusion will result when any such interaction is relatively small in effect compared with the level of dominance.

In the homozygous diallel, in the absence of non-allelic interaction, the level of dominance can be deduced using the W_r/V_r graph from the point of intersection (X) of the regression line with the W_r axis, relative to the position (Y) of $\frac{1}{4}V_{P1}$ on that axis: the necessary relationship is $\sqrt{(XY/OY)}$, where O is the origin of the W_r axis (HAYMAN 1954). With homozygosity, after correction for E_2, $\frac{1}{4}V_{P1}$ is Σuvd^2 but with parental heterozygosity it becomes

$$\Sigma[\alpha\gamma + \tfrac{1}{4}\beta(1 - \beta)]d^2 + \Sigma[\tfrac{1}{4}\beta(1 - \beta)]h^2 - \Sigma[\tfrac{1}{2}\beta(\alpha - \gamma)]dh$$

in which case it is, in general, impracticable to deduce h/d from $\sqrt{(XY/OY)}$ due to complexity in the position of Y. Also, as discussed earlier herein, the position of X will always tend to underestimate dominance when heterozygosity is involved.

Correlated allele distributions

Bias due to allele frequency correlations is similar to that for the homozygous case (HAYMAN 1954) and nothing further can be included here. It should, however, be noted that this may be a serious limitation in actual crosses since many experiments using species of economic importance will be restricted to more or less highly selected material. Further work on the details of this source of bias would appear to be necessary before more detailed conclusions are possible.

Scaling Tests

In the homozygous diallel, $(W_r - V_r)$ is constant over arrays in the absence of non-allelic interaction: if there is a significant regression of $(W_r - V_r)$ on the corresponding parental and array means, rescaling is necessary (HAYMAN 1954). In the generalised diallel there is heterogeneity of $(W_r - V_r)$ over arrays due to the heterozygous arrays, thereby vitiating any scaling test using this parameter. No suitable F_1 scaling test has been devised.

DISCUSSION

Before summarising the generalised method of diallel analysis which has been described, some discussion is required of its application to both theoretical and practical problems. The theoretical applications will be taken first since they require the briefer description in this paper.

The parental model used in deriving the foregoing analysis is representative of any population quite independently of whether its mating system is known or not. Based on this model, it is therefore possible to calculate correlations between relatives

quite independently of whether the parents involved represent a sample from a random mating population. Such generalised correlations can be applied to determine the genetic biases implicit in the normal methods of heritability estimation. The generalised treatment, thus possible, constitutes an important distinction from the special-case treatments of previous authors (e.g. WRIGHT 1935, 1952). This work is complete and will form the subject of another paper.

We turn now to the practical application of the analysis. Before proceeding further, it is necessary that one condition should be stated explicitly: namely, that any parent contributes equal samples of gametes to each family—with the homozygous parents no problem arises but with parents heterozygous at any locus the reason for the condition is obvious. In practice, the greater the number of offspring which contribute to each family-mean, the more likely is the condition to be fulfilled.

Without further qualification, the method of analysis can be applied to a diallel among a number of individuals each of which can act as both male and female parent in all the crosses (including selfing). Clearly, such conditions would limit its use to dioecious, self- and cross-compatible plants. Some modification is required before applying the method to self incompatible and/or monoecious plants and to animals. Thus, with all types of material where selfing is precluded, the analysis must be applied to crosses among groups or populations instead of among individuals. In such cases the parental groups must be derived as samples from independent populations, lines or breeds.

Obviously, this modification from parental individuals to parental groups could, in certain instances, very seriously weaken the analysis: the only generalisation possible, is that the analysis will be progressively less accurate the lower the ratio of genetic variance between parental groups to that within groups. For example, in a diallel cross among several established breeds of livestock it would, in general, be reasonable to expect the genetic variance within breeds to be considerably lower than that between breeds, in which case the diallel method could be successfully applied. In livestock breeding in particular, where experimental costs are high, the potential usefulness of the technique is very considerable since the necessary design is of such short duration compared with other types of experiment: the speed of obtaining information should, alone, offset the disadvantage of the large scale of experiment which is desirable.

Where selfing is precluded, a further alternative is worthy of brief mention. The method of analysis, as described, can be regarded as depending on a comparison between the behaviour of the parents on inbreeding and the behaviour on crossing. The inbreeding depends on comparison of V_{P1}, V_{P2} and $W_{P1/P2}$ whilst the crossing involves comparison of V_r, $\bar{V}_r$, $\bar{W}_{P1/r}$ and $\bar{W}_{P2/r}$. The inbreeding scheme used in the diallel, namely selfing gives a drop of $\frac{1}{2}h$ from the parental value in the heterozygous $\times$ heterozygous cross and this drop in the dominance component is the maximum which any form of inbreeding will achieve in one generation. If sib mating is used on the leading diagonal of the diallel table instead of selfing, the system of analysis will still apply but since the h component only drops by one-quarter in the heterozygous $\times$ heterozygous cross the solution will therefore be weaker and the coefficient of all statistics except V_{P1} will be changed. Although sib mating meets the

difficulty where selfing of individuals is precluded, nevertheless certain other difficulties are introduced. Thus, an additional generation is required for the production of sibs and when these latter are mated their progeny will not be contemporary with the remainder of the diallel except with plants, where the seed can be stored. Furthermore, if few individuals are used to produce sibs, as will be normal in animals, the genotypic frequencies of the leading diagonal (i.e. the sib matings) will be biased relative to the rest of the diallel—this bias being due to the absence of sibs produced by selfing-matings.

In organisms where a diallel among individuals is practicable, it is possible to use this technique to determine the mating structure of the population. Thus, if a sample from such a population is crossed in diallel form, the results of the analysis will indicate whether random mating is in progress or whether there is bias in favour of a particular genotypic phase. Incidentally, it should be noted that the inbreeding coefficient, f, which is determined in the course of the analysis is an empirical estimate of the level of inbreeding, as opposed to the usual type of theoretical coefficient computed from path coefficients. One great advantage gained by extention of the method to cover heterozygous crosses is the feasibility of analysing consecutive diallel crosses, the initial diallel, for instance, using homozygous material. By this means, successive analyses can be used to substantiate conclusions from earlier ones thus adding considerably to the value of this potentially powerful method of analysis.

SUMMARY

A method is given for the analysis of quantitative data from a diallel cross using as parents any type of material, homozygous or heterozygous. The method represents an extension to heterozygous crosses of the one developed by JINKS (1954) and HAYMAN (1954) for homozygous material and the theory is discussed in terms of components of variation similar to those of MATHER (1949).

The analysis provides estimates of the overall degree of dominance, of the inbreeding coefficient or degree of heterozygosity of loci showing dominance and of the allele frequency at such loci. Whether dominants or recessives are in excess can also be determined. The effect of non-allelic interaction on the statistics is discussed, together with a means of detecting such interaction.

Departures from the homozygous analysis are noted throughout and several instances are mentioned where heterozygosity, non-allelic interaction and linkage are confounded in particular statistics.

In conclusion some practical and theoretical applications of the method are considered.

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A PHENOGENETIC STUDY OF THE LOZENGE PSEUDOALLELES IN DROSOPHILA MELANOGASTER.

I. EFFECTS ON THE DEVELOPMENT OF THE TARSAL CLAWS IN HOMOZYGOTES[1]

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REEXAMINATION of a number of multiple allelic loci have uncovered cases of closely linked pseudoalleles exhibiting, at least superficially, the physiological characteristics of alleles. In several of these cases, a comparison of coupling and repulsion heterozygotes has revealed a position effect. The coupling heterozygotes appear as wild type, while the repulsion heterozygotes appear mutant (LEWIS 1951).

In considering the chromosomal distribution of genes with respect to their functions, pseudoalleles clearly represent deviations from randomness. Two general interpretations have been proposed in explanation of these deviations. One view suggests that refinement of procedures has led to an exposure reflecting upon the structure of the hereditary material (GOLDSCHMIDT 1951; PONTECORVO 1953; STADLER 1954). This view holds that the chromosome is composed of physiologically differentiated regions. Such regions may exist in alternative forms, subject to both internal and external recombination, and exhibit all of the physiological properties of alleles. According to this view, the position effect is simply a reflection of the dominance relations between alleles.

An alternative interpretation of pseudoallelism suggests that such loci represent exceptional cases of evolutionary intermediates (LEWIS 1951; GREEN 1954). This hypothesis proposes that pseudoalleles arose as duplications of single genes, and are in process of physiological and spatial distinction. In the process of physiological differentiation, such duplicates come to control adjacent steps in a biosynthetic process (LEWIS 1951, 1952). Finally, aberrational processes accompanied or followed by further physiological differentiation would lead to the establishment of a random distribution of these genes. It is proposed that such a mechanism represents a major path by which the number of genes may be increased.

The position effects of pseudoalleles are explained by the further hypothesis that the reaction sequence controlled by these genes proceeds independently on each of the homologous chromosomes. In the coupling heterozygote, the reaction path is blocked on one chromosome. However, sufficient product is formed on the homologous chromosome to yield a wild type individual. In the mutant repulsion heterozygote, the reactions are blocked on both chromosomes by the presence of mutant genes. A further modification of this hypothesis permits some diffusion of intermediates between homologous chromosomes (LEWIS 1954).

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Thus, two hypotheses are proposed in explanation of the phenomenon of pseudoallelism and associated position effects. This paper presents one aspect of a program of studies designed to contribute evidence towards the resolution of this problem.

The three lozenge pseudoalleles in *Drosophila melanogaster* represent a particularly favorable series for study (GREEN and GREEN 1949). A large number of possible genic combinations are available. Pleiotropy is exhibited and several variables may be studied. The mutant phenotype includes abnormalities in the external shape and pigmentation of the eye (GOTTSCHEWSKI 1936; OLIVER 1947; GREEN 1948); abnormalities in the various structural components of the ommatidia (CLAYTON 1952); infertility of females (OLIVER and GREEN 1944; ANDERSON 1945); antigenic changes (CHOVNICK and FOX 1953); and abnormalities in the structure of the distal segment of the leg, the tarsus (CUMMINGS 1946; CHOVNICK 1953).

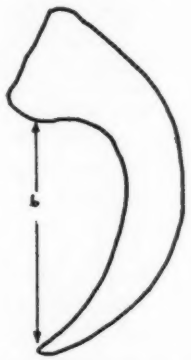


FIGURE 1.—Diagram of a normal shaped tarsal claw. (b) Represents straight line measurement used as estimate of claw size.

The two major structures of the tarsus are the claws and pulvilli. Both structures are affected by the lozenge mutants. The claw anomalies range from a reduction in claw pigmentation through reduction in size of claws to complete absence of claws. The effect on the pulvillus, a glandular structure, appears to parallel the claw anomaly.

The structural changes in tarsal claws accompanying change in dosage and position of the lozenge pseudoalleles were studied. The dosage study in homozygotes is reported in this paper. Claw effects accompanying changes in position are to be reported separately.

MATERIALS AND METHODS

Flies were cultured in half-pint milk bottles on the standard corn meal–molasses–agar medium fortified with yeast extract and seeded with living yeast prior to use. The cultures were maintained at 27°C in a constant temperature incubator. Each culture was started by the addition of two pairs of males and virgin females. Parents

were removed on the seventh day, and only the first 10 to 20 mutant females to emerge were examined.

Examination of heterozygotes and comparison with homozygotes was considered essential for the study. Since lozenge is sex-linked (only female heterozygotes can be obtained), the entire study was confined to females, removing possible sex-influenced effects in such comparisons. The data collected on any one genotype was accumulated over a period of time, and includes a minimum of seven replicates.

Legs were removed, placed on albuminized slides, and the tarsal claws were examined under the microscope. The measurement *b* (fig. 1) was made with a Bausch and Lomb filar micrometer eyepiece at 537.5 magnifications. A number of difficulties in the measurement will be discussed below. It should be noted that possible subjective influence in the measuring procedure was removed in the following fashion: Matings were coded and individuals to be examined were recognized by code number. The slides were prepared, recorded and randomized. The labor was divided between the authors in such a fashion that any pair of legs under observation could not be related to any other pair, or to any genotype, without conscious effort.

DERIVATION OF STRAINS

In studying the effects of genic substitution at specific loci, the ideal procedure would vary only the genes of interest. Although such a procedure is technically impossible, an approach to this ideal was attempted.

A series of seven stocks possessing the sex-linked pseudoalleles of lozenge; *BS*, *46*, and *g*, in all possible combinations were made available by M. M. GREEN. These stocks were subjected to a series of crosses designed to render them coisogenic with a highly inbred wild strain of the Oregon R-I series of J. SCHULTZ.

In the following description of the derivation of experimental strains, the symbol, *lz*, will be used to represent the lozenge regions. The derivation of all strains was accomplished in two steps: (1) The transfer of the lozenge combinations from diverse X chromosomes to the Oregon R-I series X chromosome. This step was accomplished by use of marker genes closely linked to lozenge, and recovering crossovers from heterozygous females. (2) Substitution of Oregon R autosomes was accomplished by use of dominant marked inversions. During this step, the Oregon R X chromosome was introduced into each strain (figs. 2 and 3).

In this fashion six balanced strains were established, each essentially differing from the inbred Oregon R strain only with respect to the lozenge region of their X chromosomes. The following lozenge combinations were established in the described derivation: *BS46g*, *BS46+*, *BS+g*, *BS++*, *+46+*, *++g*. The Oregon R strain served as wild type. The combination *+46g* used in this study was derived by crossing over in heterozygotes *+46+/++g* possessing the isogenic background.

Following their derivation, these *ClB* balanced strains were maintained by brother-sister mating. A number of possible sources of deviation from coisogenicity are inherent to the procedure and should be noted: (1) The X chromosomes of the stocks may differ with respect to the 12 crossover unit segment between singed and vermilion which includes the lozenge region. However, X chromosomal crossovers outside of

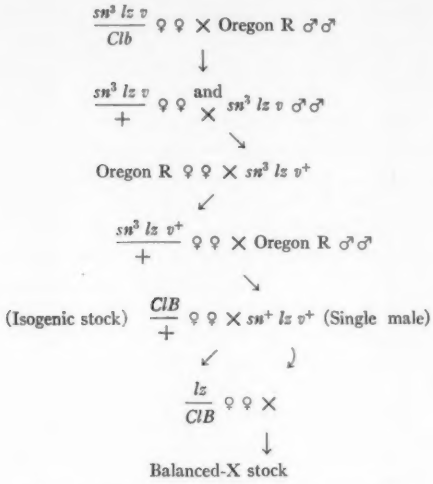

$\frac{sn^3\ lz\ v}{Clb}$ ♀♀ × Oregon R ♂♂
$\frac{sn^3\ lz\ v}{+}$ ♀♀ and × $sn^3\ lz\ v$ ♂♂
Oregon R ♀♀ × $sn^3\ lz\ v^+$
$\frac{sn^3\ lz\ v^+}{+}$ ♀♀ × Oregon R ♂♂
(Isogenic stock) $\frac{ClB}{+}$ ♀♀ × $sn^+\ lz\ v^+$ (Single male)
$\frac{lz}{ClB}$ ♀♀ ×
Balanced-X stock

FIGURE 2.—Transfer to common X-chromosome.

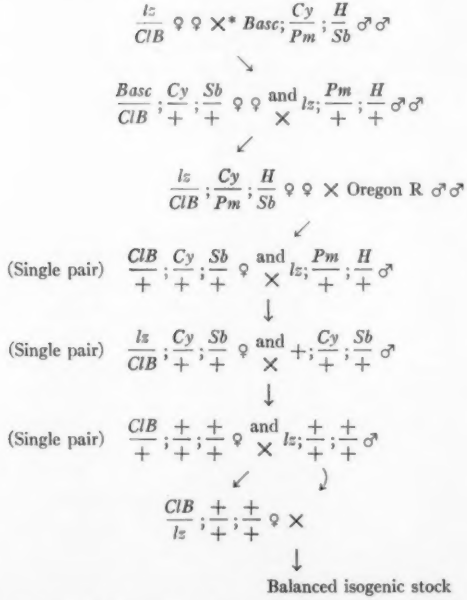

$\frac{lz}{ClB}$ ♀♀ ×* $Basc$; $\frac{Cy}{Pm}$; $\frac{H}{Sb}$ ♂♂
$\frac{Basc}{ClB}$; $\frac{Cy}{+}$; $\frac{Sb}{+}$ ♀♀ and × lz; $\frac{Pm}{+}$; $\frac{H}{+}$ ♂♂
$\frac{lz}{ClB}$; $\frac{Cy}{Pm}$; $\frac{H}{Sb}$ ♀♀ × Oregon R ♂♂
(Single pair) $\frac{ClB}{+}$; $\frac{Cy}{+}$; $\frac{Sb}{+}$ ♀ and × lz; $\frac{Pm}{+}$; $\frac{H}{+}$ ♂
(Single pair) $\frac{lz}{ClB}$; $\frac{Cy}{+}$; $\frac{Sb}{+}$ ♀ and × +; $\frac{Cy}{+}$; $\frac{Sb}{+}$ ♂
(Single pair) $\frac{ClB}{+}$; $\frac{+}{+}$; $\frac{+}{+}$ ♀ and × lz; $\frac{+}{+}$; $\frac{+}{+}$ ♂
$\frac{ClB}{lz}$; $\frac{+}{+}$; $\frac{+}{+}$ ♀ ×
Balanced isogenic stock

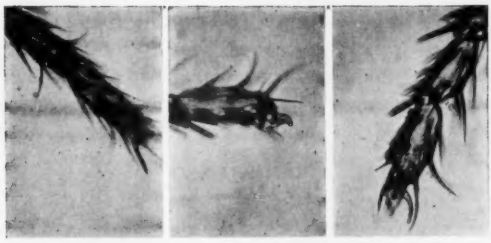
FIGURE 3.—Substitution of Oregon-R Autosomes and Y Chromosome.

*Complete formula: $sc^{sv}B\ In\text{–}S\ w^a\ sc^8$; Cy/Pm, ds^{33k}; $H/In(3R)Mo\ Sb\ sr$.

this region may lead to other differences. (2) Heterogeneity of autosomes (2 and 3) may have resulted from crossing over between Oregon R chromosomes and "marker" chromosomes during the derivations, particularly in the unsupressed regions of these chromosomes. (3) The fourth chromosome was not controlled during the derivation. However, during the derivation there were four outcrosses to Oregon R rendering it probable that the mutant stocks possess Oregon R fourth chromosomes. (4) Spontaneous mutation with visible effect since the derivation has been noted and selected out. No control over non-visible mutation is possible.

CLAW MEASUREMENTS

Preliminary attempts to measure the size of tarsal claws met with several problems. Due to the small sizes involved, the claws must be measured under the compound microscope. When a leg is placed on a slide, it is impossible to orient the claw



A B C

PLATE 1.—A, B, and C shaped tarsal claws, 450×.

in precisely the same position each time. Since positions may vary from claw to claw, some measures would be direct estimates of the straight line distance *b* (fig. 1), and others would be estimates of various functions of *b*. Assuming all claws in all genotypes to be equally capable of such variation in position, the error incurred would not be significant. Moreover, a further difficulty arises when it is noted that claw shape may vary. Three distinct categories of claw shape were recognized. The three categories were labeled A, B, and C (plate I). Group C claws are the normal curved claws, and are also the most common type found in this study. Group A claws are straight, and group B represents a heterogeneous class of abnormally shaped claws. The latter group share in common the feature of being, almost exclusively, very short claws. It should be understood that these categories were fixed arbitrarily. Considerable intergrading does occur, particularly between the classes A and C.

The problem of claw position variation leads to a further difficulty. Certain of the positions preclude measurement of the claw length. Attempt to adjust the position of any claw would, for the sake of consistency, force adjustment of all claw positions. Such a laborious procedure would seriously limit sample size. Assuming that the probability of any claw to be unmeasurable is a constant, regardless of genotype, omitting such claws from the data would incur no sizable error. Time would be more profitably spent in increasing sample size.

The third problem concerns itself with the number of claws on any one individual. Does an adequate estimate of the effect per fly require measurement of the twelve claws per fly?

A preliminary study was made to gather information bearing on the following questions: To what extent does variation in claw shape lead to error in estimation of the effect of genotype on claw size? Does omission of unmeasurable claws lead to a similar error? Does an adequate estimate of the effect per fly require examination of all legs of each fly?

At the time of these experiments, the genotype *+46g* was in process of derivation, and was consequently omitted. All six legs were examined, claws were measured, classified as to shape. Measurements were recorded in terms of units on the scale of the filar micrometer (37.1 units = 0.01 mm at 537.5 magnifications). Claws were subsequently graded as follows: 0–50 (1), 51–75 (2), 76–100 (3), and increasing by units of 25. The largest claws ever found fell in the category 176–200 (7). Since the wild type possesses two claws per leg, two claws were expected on each leg. A missing claw was graded zero. A claw value per leg was obtained by summing the values for the four claws on each pair of legs and dividing by two.

Examination of individuals of the various genotypes revealed that two of the genotypes, *BS46+* and *BS46g*, rarely possess claws (table 1). Considering only those genotypes with appreciable incidences of claws, it is noted that the distribution of abnormal claw types (A and B) from leg to leg is not random. Only 0.21 of these claws were on the third legs, and the frequency increases from posterior to anterior (chi square = 10.1, d.f. = 2). Furthermore, the pooled frequencies of abnormal claws among the genotypes are quite low. With one exception, this value is not higher than 0.045. The exceptional case is that of *BS+g* where 0.127 of the claws examined were of abnormal shape, and almost all of these were small claws of type B. However, the incidence of abnormal claws in this genotype does not markedly influence the estimate of the mean claw value per leg (table 2). In spite of the fact that most of the abnormal claws in this genotype were on the first and second legs, estimates of the mean using the first, second, or third legs are not significantly different. In

TABLE 1

Distribution of claw shapes, unmeasurables, and missing claws on legs of homozygotes

Homozygous genotype	Claw types												Claw shapes			% Abnormal claw shapes	0 claws	UNM claws	% UNM claws	No. sites with claws
	A			B			UNM			0										
	Leg			Leg			Leg			Leg										
	1	2	3	1	2	3	1	2	3	1	2	3	A	B	C					
+++	2	1	1	0	0	0	43	27	35	0	0	2	4	0	585	0.68	2	105	17.8	589
++g	4	2	0	0	0	0	26	23	18	18	14	14	6	0	393	1.50	46	67	16.8	399
+46+	8	4	0	1	0	1	13	16	18	19	15	7	12	2	291	4.50	41	47	15.4	305
BS++	2	1	0	3	1	3	11	8	7	62	60	69	3	7	208	4.50	191	26	11.9	218
BS+g	1	0	0	11	7	4	5	6	6	42	49	48	1	22	157	12.70	139	17	9.4	180
BS46+	1	1	1	0	1	3	0	1	0	125	125	124	3	4	5	58.30	492	1	8.3	12
BS46g	1	0	0	1	0	1	0	1	0	166	166	167	1	2	3	50.00	498	1	16.6	6

TABLE 2

Estimates of mean claw value/leg for first, second, and third legs of homozygotes

Homozygous genotype	Leg			F	d.f.
	1	2	3		
+++	8.35	8.92	9.32	10.80	2,147
++g	6.73	7.39	7.76	4.76	2,111
+46+	6.29	7.23	8.01	5.65	2, 87
BS++	3.98	4.19	3.67	0.63	2, 102
BS+g*	3.87	3.43	4.33	1.30	2, 78
BS46+	0.12	0.10	0.14		
BS46g	0.10	0.08	0.05		

* Excluding B claws: 4.07, 3.61, 3.95.

addition, estimate of the genotype mean excluding abnormal claws, leads to no appreciable change. From these observations, it is seen that variation in claw shape does not constitute a serious problem for estimation of claw length. No apparent error is introduced by including the abnormal claws. Restriction of observations to the third pair of legs would remove most of the abnormal claw shapes, and further reduce any error in estimation of claw size.

Distribution of unmeasurable claws (UNM) and missing claws from leg to leg shows no deviation from randomness (table 1). However, the frequency of unmeasurable claws decreases as the genotype mean decreases. This difference, a maximum of approximately nine percent, will tend to reduce the estimates of the mean per genotype, and the reduction will increase with decreasing means. However, the error incurred will not alter the relative positions of the various genotypes. In the absence of evidence that omission of unmeasurables would lead to a significant differential error in estimating means, this procedure was followed in the remainder of the study.

From consideration of the distribution of variant claw shapes, it was found that restriction of study to the third pair of legs would considerably reduce error from this source. Further evidence supporting such a restriction is seen in table 2. The mean claw values of +++ for the three leg pairs increase from anterior to posterior. With substitution of mutant alleles, the gradient effect disappears in the genotypes with small claws. Thus the third legs appear to be more sensitive to mutant gene substitution. Further implications of the gradient effect are discussed below. The remainder of the study was restricted to estimates based upon third leg measurements.

HOMOZYGOTE ANALYSIS

AUERBACH (1936) has described the development of the thoracic appendages of *Drosophila melangoaster*. These observations indicate that the period of greatest developmental activity occurs earlier in the first leg buds than in the second, and the third leg buds lag far behind. The observation (table 2) of a claw size gradient in the wild type disappearing with increased mutant gene substitution supports the following interpretations of gene action.

TABLE 3

Means and variances of third legs of homozygotes

Homozygous genotype	Direct estimates		Corrected estimates		Adjusted mean	Sampling variance	n
	Mean	Variance	Mean	Variance			
+++	8.78	5.10	—	—	10.34	0.056	91
++g	7.35	2.44	—	—	8.91	0.015	162
+46+	6.69	4.89	—	—	8.25	0.034	143
+46g	5.63	5.06	—	—	7.19	0.043	119
BS++	3.38	5.36	3.27	6.34	4.83	0.040	161
BS+g	3.00	4.51	2.88	5.48	4.44	0.033	174
BS46+	0.65	0.23	−0.49	4.86	1.07	0.0086	81
BS46g	0.31	0.22	−1.56	4.86	0.00	0.012	98

Both wild and mutant pseudoalleles contribute towards the conversion of a substrate to a product involved in claw production. The different alleles control the rate at which the substrate is converted into product. The genotype, +++, may be considered as most efficient in this conversion. Claw size on any leg results as a function of substrate availability at a critical point in development, and differs for each leg. However, with decreasing efficiency of substrate conversion, the gradient effect would be expected to diminish (++*g* and +*46*+). Continued decrease in efficiency of this conversion would lead to a gradient below the level of resolution (*BS*++ and *BS*+*g*).

An alternative interpretation would consider the lozenge mutants as inhibitors of claw development. ANDERS (1948) observations on the effects of lz^{el} in the presence of the *tra* gene suggest that the lozenge effect is quite late in development. In the presence of the wild pseudoalleles of lozenge, substrate availability at periods of maximum developmental activity lead to the gradient effect. The lozenge mutants, acting late in development, lead to the production of an inhibitor, or divert the substrate into another path. Such action asserts its greatest influence on later developing legs, and leads to a depression of the gradient in claw size.

Table 3 contains a summary of the data collected on tarsal claws of the third legs of homozygotes. Histograms of the distribution of claw values per fly for the various genotypes are given in figure 4.

Complete distributions are found for those genotypes possessing larger claws; +++, ++*g*, +*46*+, and +*46g*. With decreasing means, the genotype distributions present an increasing degree of truncation at the zero value as exhibited by *BS*++, *BS*+*g*, *BS46*+, and *BS46g*. On either of the schemes of gene action described above, the truncation may be attributed to the operation of a developmental threshold. Assume a certain minimal quantity of gene product is necessary for claw development. Below this level, no claw is produced. Above threshold, the mean claw value represents an estimate of the average amount of product attributable to the genotype. Variation within any one genotype may be attributed to environmental factors.

Estimation of means and variances for the complete distributions were made

directly from the data. However, direct estimation of the means of the truncated distributions would lead to error increasing with degree of truncation. Such estimates would be higher than the true means. Estimates of the variance of truncated distributions would be lower than the true variance. Following the method of IPSEN (1949), estimates of the parameters of the truncated distributions were obtained. In two of the distributions, *BS++* and *BS+g*, only small portions of the distribution

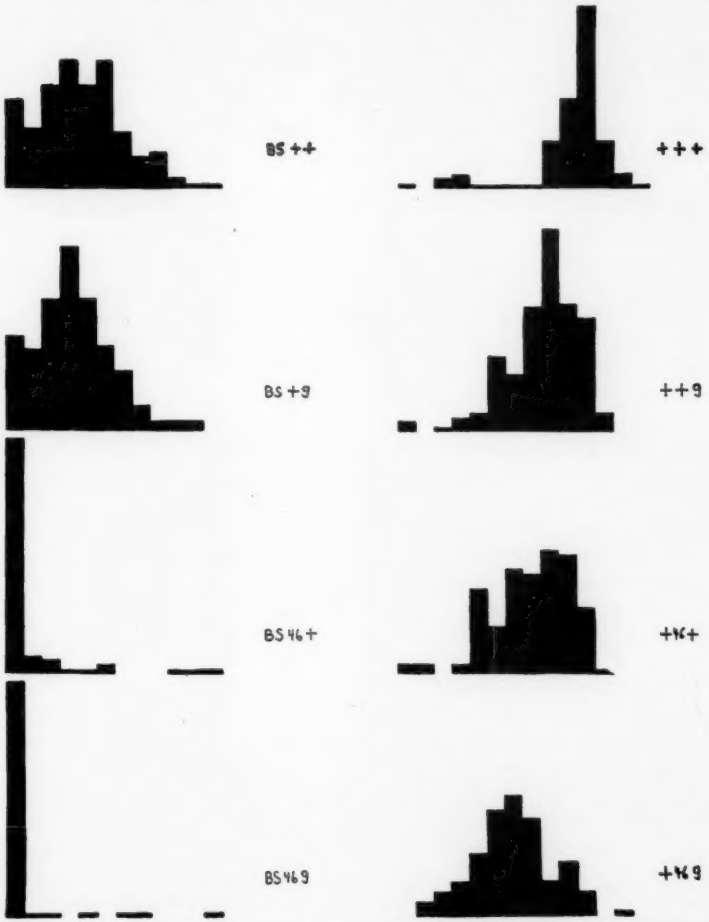


FIGURE 4.—Frequency histograms of third leg claw values/fly for the indicated genotypes. Horizontal axis indicates claw values/fly: 0, 0.1–1.0, 1.1–2.0, 2.1–3.0, 3.1–4.0, 4.1–5.0, 5.1–6.0, 6.1–7.0, 7.1–8.0, 8.1–9.0, 9.1–10.0, 10.1–11.0, 11.1–12.0. Vertical axis indicates number of flies in each category. Zero column in *BS46+* and *BS46g* distributions should be higher than indicated.

fall below the threshold. Truncated means and variances were estimated, and used to obtain estimates of the true means and variances (table 3, Corrected Estimates). In the distributions, *BS46+* and *BS46g*, only small portions of the distributions fall above the truncation point. Estimation of variance of these distributions from the small samples above the truncation point would lead to sizable error. Assuming that variation in all genotypes is due to random fluctuations in common environmental factors, the variance should be independent of the mean. No relationship between mean and variance is apparent in these data. The average variance of all distributions was used as an estimate of variance for the extremely truncated distributions. Using the truncated means and the average variance estimate of the true variance, the true means of the truncated *BS46+* and *BS46g* were obtained (table 3, corrected estimates). To remove negative means, the *BS46g* mean was set at zero, and all other means adjusted by adding 1.56 (table 3, adjusted means). The adjusted means represent estimates of the amount of gene product attributable to the genotype. These estimates have been corrected for the threshold at the truncation point and rated on a positive scale.

Estimates of the sampling variance of the mean were obtained directly for the complete distributions. The method of IPSEN (1949) was used to estimate the sampling variance of the means for the truncated distributions (table 3, sampling variance). The adjusted means associated with homozygous genic substitution separately and in compounds reveal a cumulative effect suggesting additivity of action of the lozenge loci. A test for additivity of gene action, making use of the adjusted means and their sampling variances, is provided by HOUSE (1953). A modification of this method, when applied to these data, rules out the hypothesis of additivity.

The cumulative effects seen in the present data are consistent with two categories of mechanisms of gene action. One possibility views the lozenge loci as physiological duplicates, each locus operating upon the same or similar substrate(s), and producing the same or similar product(s). The different alleles at these loci may control the rates of the reaction, or the product specificity for claw production, and thus govern the relative contribution of each locus to claw size. Although such a mechanism of gene action would predict an additive effect, deviations from additivity due to factors external to the lozenge loci are possible.

An alternative hypothesis suggests that these loci control successive steps in a sequence of reactions (LEWIS 1951; GREEN 1954). These data would support such a view, if it is further assumed that the various alleles at these loci control the rates of the individual reactions. Information concerning the possible localization of the reaction sequence to the site of the genes in the chromosomes may be derived from an examination of heterozygotes.

DISCUSSION

The spatial distinction, physiological similarities, and position effects exhibited by pseudoallelic loci have been interpreted in two general fashions. One view supported by LEWIS (1951) and GREEN (1954) consists of three features: (1) pseudoalleles represent duplications of genic material, (2) physiological differentiation, associated with or following upon duplication, leads to the association of such loci with successive

and distinct steps in a sequence of reactions, and (3) the reactions occur localized to the site of the genes in the chromosomes.

GREEN (1954) in a study of a series of vermilion mutants reported that they could be classed into two groups on the basis of interaction with a non-allelic suppressor of vermilion. One group of mutants, including v^1, exhibit wild type eyes in the presence of the suppressor, and consequently are labeled v^s mutants. Another group, including v^{36f}, are vermilion in compounds with the suppressor, and are labeled v^u mutants. It was further found that v^1 and v^{36f} are not alleles, but closely linked pseudoalleles. On the basis of these observations, GREEN suggested that all v^s mustants are alleles of v^1, and all v^u mutants are alleles of v^{36f}. BARISH and FOX (1955) reported that v^{48a}, an unsuppressed mutant, exhibited recombination with v^{36f} and not with v^1. These observations indicate that v^{48a}, an unsuppressed vermilion, is allelic to v^1, a suppressed mutant. Further evidence of overlapping physiological effects is indicated from a study of the antigenic effects of the vermilion pseudoalleles (BARISH and FOX 1955). It was noted that the mutants v^1, v^{36f}, and v^{48a} share, in common, the antigenic component V-1. The mutants v^{36f} and v^{48a} possess a component, V-2, which is absent in v^1 mutants. A third component, V-3, possessed by v^{48a}, is absent in v^1 and v^{36f} homozygotes. These observations on allelic relations and overlapping physiological effects of the vermilion mutants do not support the view that pseudoallelic loci control separate and distinct steps in a sequence of reactions.

CHOVNICK and FOX (1953) found evidence supporting an interchromosomal interaction in the production of an antigenic component associated with the lozenge pseudoalleles. The component, L-3, was found to be present in both coupling[1] and repulsion heterozygotes involving the lozenge mutant pseudoalleles *BS* and *46*, and not present in either homozygote. Such evidence is not consistent with the third aspect of the hypothesis suggesting gene action localized to the chromosomes. In contrast to the position effects associated with morphological effects of pseudoallelic loci in Drosophila, no position effect is seen in antigenic specificities associated with either the vermilion mutants (BARISH and FOX 1955) or the lozenge mutants (CHOVNICK and FOX 1953). This apparent discrepancy suggests that the observations are being made on different levels of gene action. Priority with respect to validity of conclusions concerning the primary action of these genes awaits further study. The mechanism of gene action, in any case, should be capable of explaining all of the observations.

The antigenic effects of the lozenge pseudoalleles permitted two categories of mechanisms of gene action: (1) the loci were each associated with successive steps in a sequence of reactions and (2) the loci were concerned with conversion of the same or similar substrate(s) into the same or similar product(s). The present observations, concerned with effects of homozygous substitution of mutant pseudoalleles of lozenge upon the development of tarsal claws, are wholly consistent with the observations on antigenic effects. In the study of antigenic effects, it was noted that two components, L-1 and L-2, were associated with both of the mutants *BS* and *46*, while only the L-1 component could be detected in the presence of *g*. In considering the effects on the development of tarsal claws, homozygous substitution of *g* results in a lesser depression of claw size than does either *BS* or *46*. A number of possibilities exist for cor-

relating the antigenic and morphologic effects, but it is too soon for more than speculation.

A second interpretation given to the effects associated with pseudoallelic loci suggests that chromosomes consist of physiologically differentiated regions. These regions or units may exist in alternative forms, and are subject to both internal and external recombination. Since these differential regions are the physiological units of heredity, all of the physiological attributes of genes should refer to them. Indeed, the position effects associated with pseudoalleles are to be expected as examples of the physiological relations between alleles (GOLDSCHMIDT 1951).

In comparing the LEWIS hypothesis with that of GOLDSCHMIDT, there appears a similarity between the two views. Omitting the duplication and evolutionary intermediate aspect of the LEWIS hypothesis, both envision a similar structure to the hereditary material. The LEWIS hypothesis then resolves itself as a specific case of the GOLDSCHMIDT concept of the hereditary material. It should be expected that different physiological regions or units may exhibit different internal activities, one type being similar to that proposed by LEWIS. Moreover, in some cases, the gene defined on the operation of a breeding experiment may correspond to the physiological unit, while in others, it may be only part of such a unit.

The position that pseudoalleles are duplicates and evolutionary intermediates is based upon the association of several cases of pseudoallelic loci with salivary chromosome doublet structures. BRIDGES (1935) suggested that such structures may be single band duplications that arose by unequal crossing over. This view implies that the chromosomal regions occupied by pseudoallelic loci constitute special cases, and do not reflect upon the organization of most of the hereditary material. However, an examination of the revised map of the salivary gland X chromosome of *Drosophila melanogaster* (BRIDGES 1938; BRIDGES and BREHME 1944) reveals that 65% of the bands are associated with doublet structures. Adjacent single bands with similar morphology, and single bands adjacent to and morphologically similar to bands associated in doublets, may be interpreted as duplications which have become somewhat further differentiated. Inclusion of such bands in the count of doublet bands suggests that the pseudoallelic organization is the rule rather than the exception.

Assuming the duplication aspect of the LEWIS hypothesis to be correct, a major path for the production of new genetic material is seen. Unequal crossing over associated with or followed by some physiological differentiation leads to a chromosome organized into physiological units, each unit internally coordinated, and in control of some process. One type of internal coordination may be that of a linear sequence of reactions as proposed by LEWIS. Other types of internal organization should be expected. Further physical separation and differentiation would take place over a long period of time. At any one time, the pseudoallelic organization would represent a typical organization of the hereditary material.

No revision of experimental approach is warranted from the above consideration. The basic tool in any study of heredity is the breeding experiment, the smallest common denominator of which is the unit of recombination. However, in attempting to understand the manner in which heredity asserts itself in cell physiology, studies of the physiological units and their internal organization will be of some value.

Question has been raised as to the criteria for defining such physiological units (LEWIS 1954). The physiological unit, like the breeding unit is an operational one. It is suggested that the operational approach be followed in each case. Pseudoallelism defines the empiric unit until further definition is justified.

SUMMARY

A series of coisogenic strains of *Drosophila melanogaster* were derived. These strains differ essentially in constitution of the lozenge region of the X chromosome. All homozygous combinations of mutant and wild pseudoalleles of lozenge (*BS*, *46*, and *g*) were examined with respect to their effects on the development of the tarsal claws. A cumulative, but not additive, effect was noted suggesting two categories of mechanisms of gene action: (1) all of the loci are physiological duplicates and (2) the loci control the rates of successive steps in a sequence of reactions. Implications of these results for interpretation of pseudoallelism are discussed.

ACKNOWLEDGMENTS

The authors express their appreciation of the advice, discussion, and cooperation of DR. A. S. FOX of Michigan State University, and to DR. M. M. GREEN of the University of California at Davis for making available the various combinations of lozenge pseudoalleles used in the study.

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INHERITANCE IN THE CARNATION, DIANTHUS CARYOPHYLLUS. V. THE CHEMISTRY OF FLOWER COLOR VARIATION, II

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IN the first article of this series, GEISSMAN and MEHLQUIST (1947) described the chemical effects that accompanied alterations in the genetic constitution of carnations with respect to the factors *S*, *R* and *M*. It was found that only *R* affected the degree of nuclear hydroxylation of the anthocyanin pigments of the flowers. Cyanic forms that contained *R* in homozygous or heterozygous condition contained cyanidin glycosides; the homozygous recessives (*rr*) contained pelargonidin derivatives. At the time of that study the number of genetically analyzed non-cyanic forms was limited, and of the whites and yellows only *rr* specimens were at hand. It was found that these white and yellow *rr* flowers (which contain no anthocyanins) did contain the flavonol kampferol, the hydroxylation pattern of which is the same as that of pelargonidin. Furthermore, from a crimson form ("Woburn," *Yy*^{l} *IiA*^{var} *aSSRrmm*)[1] were isolated kampferol and quercetin—the latter the flavonol corresponding in hydroxylation pattern to cyanidin, the anthocyanidin of *R*– plants.

These results afforded a tentative basis for the suggestion that *R* was not uniquely associated with the anthocyanidin structure but was a factor concerned in the hydroxylation pattern of the "B-ring" of both the anthocyanins and flavones.

The present study has extended the early observations, and includes an examination of the flavonoid constituents of (a) seven *rr* cyanic flowers (reds and pinks) and (b) nine whites, of which eight were *rr* whites and one an *R*– white.

MATERIALS AND METHODS

Cyanic forms

Commercial carnations were purchased in the market. The following, with the genotype (for flower color) so far as it is known,[2] were used (table 1).

The following description of the manner in which one of the varieties was treated is typical of the procedure that was followed:

The petals (1450 g) of Chalfonte were removed by cutting off the colored portion outside of the calyx, and macerated with methanol in a Waring Blendor. The resulting slurry was filtered through Celite and the marc reextracted with methanol four times. The combined extract was concentrated to about one liter, 800 ml of 5% sulfuric acid was added, and the resulting solution concentrated by boiling (steam bath) until about 500 ml of methanol had been removed. The deep red solution was filtered (see below) and extracted with ether. Removal of the ether by distillation

[1] The genetic designations are described by GEISSMAN and MEHLQUIST (1947).

[2] We are grateful to PROF. G. A. L. MEHLQUIST for the information concerning the genetic constitution of these flowers.

TABLE 1

Color	Variety	Color genotype
Deep pink	Chalfonte	*Y–I–A–S–rrM–*
Salmon	Ida	*Y–I–A–ssrrmmsusu*
Red	Tom Knipe	*Y–I–A–S–rrmm*
Light pink	Light Pink Peter Fisher	*Y–I–A–S–rrM–Su–*
Deep pink	Deep Pink Peter Fisher	*Y–I–A–S–rrM–susu*
Pink	Pink Spectrum	*Y–I–A–ssrrmm*
Red	Red Spectrum	*Y–I–A–S–rrmm*

left a crystalline residue that weighed 4.0 g. This material appeared to be a sapogenin, since it gave a magenta color with acetic anhydride-sulfuric acid (Liebermann-Burchard reaction); but because it gave negative tests for flavonols, it was not examined further at the time.

The gummy residue from the filtration of the hydrolyzate was extracted exhaustively with ether, affording 8.62 g of a brownish-yellow solid. This was acetylated by boiling 1.5 g with acetic anhydride (15 ml) and sodium acetate (1.5 g). The resulting flavonol acetate (0.75 g) formed cream-colored needles after recrystallization from ethanol, and had m.p. 185–186.5°C.

In the same way, crude flavonol acetates were obtained from each of the seven flowers. These had the following properties (table 2):

The results of the mixed melting-point determinations afforded no unambiguous answer to the question of the identity of the flavonol acetates, probably because of their contamination with other substances. Since attempts to "purify" the acetates by recrystallization could have resulted in the removal of small amounts of quercetin or kampferol, recrystallization would have vitiated the whole purpose of this approach, which was to determine whether kampferol or quercetin, or both, were present in the flowers.

Paper chromatography

Ten mg of each of the crude acetates was dissolved in a mixture of 1 ml of methanol and 1 ml of water to which a drop of concentrated hydrochloric acid was added, and

TABLE 2

Variety	M.p. of crude flavonol acetate	M.p. of mixture with	
		Kampferol tetraacetate*	Quercetin pentaacetate*
Chalfonte	185–186.5°C.	180–185°C.	170–175°C.
Ida	185–190°	179–186°	173.5–177°
Tom Knipe	188–189°	180–185°	172–180°
L. P. Peter Fisher	182–185°	179–183.5°	171–180°
D. P. Peter Fisher	183–187°	175–184°	167–185°
Red Spectrum	185–188°	181–187°	170–184°
Pink Spectrum	176–192°	165–181°	171–185°

* m.p. of kampferol tetraacetate, 182°C; m.p. of quercetin pentaacetate, 195°C.

the solution refluxed for one hour. The cooled solution was applied to Whatman No. 1 paper (25 μl) and the chromatogram developed with butanol (1 part)–27% acetic acid (1 part). Authentic samples of quercetin and kampferol, and a sample of the flavonol acetate from "Woburn," treated as above, were run on the same sheet.

The results were clear and unambiguous. Kampferol was clearly visible in all of the pink and red genotypes, and quercetin was absent from these but clearly visible in the "Woburn" sample.

Whites

Eight "white" clones were furnished by PROF. G. A. L. MEHLQUIST, whose breeding experiments had established their genotypes as follows:

Clone no.	*Genotype*
49040-1	*yyI–A–S–R–M–*
49041-5	*YYIIaaSSrrmmSuSu*
51622-1	*a*—white from selfing 49041-5; same genotype
51622-7	*a*—white from selfing 49041-5; same genotype
49041-6	*a*—white, sister seedling to 49041-5; probably same genotype
49086-5	*yyIIAASSrrMM*
49086-8	*yyIIAASSrrMM*
51632-3	*yyIiA–S–rrMM*

A sample of the petals of each of these[3] was dried in a vacuum desiccator over sulfuric acid and ground to a powder. A 100 mg portion was extracted with hot methanol, and a sample of the methanol extract, along with the marc, hydrolyzed by heating with 5% sulfuric acid for 2 hours. The cooled solution was extracted four times with 1 ml portions of ethyl acetate and the extract concentrated in a gentle stream of air to about 0.5 ml. Ethanol was added to make the volume 1 ml. Samples of about 10 μl of the final solution were chromatographed on Whatman No. 1 paper along with authentic specimens of quercetin, kampferol and apigenin. After drying, the chromatograms were sprayed with 2% alcoholic aluminum chloride to develop the brightly green-yellow fluorescence characteristic of 3-hydroxyflavones.

All of the white clones contained kampferol, *but only 49040-1 contained quercetin as well.* Apigenin was not observed to be present in these whites.

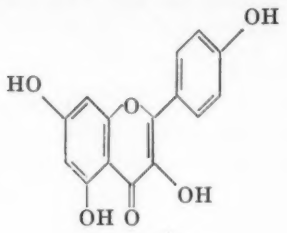
It is of interest to note that chromatograms of the original methanol extracts of the *y*—whites (49086-5, 49086-8, 51632-3) and the *a*—whites (51622-1, 51622-7, 49041-6) were clearly distinguishable from each other. The chemical differences between these two kinds of flowers cannot yet be described, but the identification of the components of these (complex) chromatograms will be undertaken in an effort to determine the chemical effects of the *A* and *Y* factors.

DISCUSSION

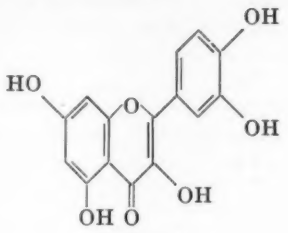
The present findings support the suggestion made earlier that the *R* factor in carnations controls the degree of oxidation of the B-ring in the flavonoid constituents of

[3] PROF. B. LENNART JOHNSON of the Division of Ornamental Horticulture of U.C.L.A. was kind enough to grow the plants from rooted cuttings furnished by PROF. MEHLQUIST.

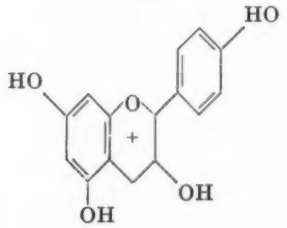
the flowers. The effect of *R* is shown in the following summary:



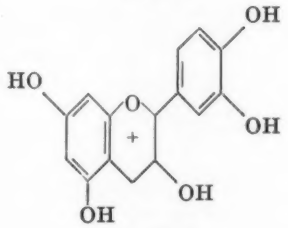
kampferol



quercetin



pelargonidin
rr



cyanidin
R–

Since kampferol is found along with quercetin in *R–* flowers, it is surprising that cyanidin alone is present in the cyanic forms that carry *R–*. Indeed, this point now requires reexamination. In the early work (GEISSMAN and MEHLQUIST 1947) the nature of the cyanic pigmentation was established by the application of the color tests of ROBINSON and ROBINSON (1931) to petal extracts. It was remarked at that time that while the presence of cyanidin as a contaminant of pelargonidin could be detected by these color tests, small amounts of pelargonidin could escape detection in the presence of cyanidin. With the aid of chromatographic methods, the presence of either pelargonidin or cyanidin as a concomitant of the other can now be detected. Experiments to reexamine this point are to be performed.

The *R* factor of carnations resembles in its effects the *M* factor of the snapdragon (*Antirrhinum majus*), as described by GEISSMAN, JORGENSEN and JOHNSON (1954), JORGENSEN and GEISSMAN (1955a, b), and GEISSMAN and HARBORNE (1955). This factor controls hydroxylation in the B-ring of the flavonoid pigments of this flower, acyanic *M–* flowers containing luteolin and apigenin, cyanic *M–* flowers luteolin, apigenin, quercetin and cyanidin, while *mm* flowers contain only apigenin if acyanic, and apigenin, kampferol and pelargonidin when cyanic.

The present results further emphasize the importance of examining the noncyanic pigments in studies of the chemical effects of genetic changes. Carnations and snapdragons both contain factors whose phenotypic expression appears to be an alteration of the structures of the anthocyanin pigments of the pink to magenta flowers, but which in reality exert a structural control at another level of synthesis.

It is hoped that further studies of selected carnation genotypes will make it possible to define more exactly the nature of the chemical reactions at which these and other factors exert their control.

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THE GENETICS OF RESISTANCE OF SACCHAROMYCES TO LITHIUM CHLORIDE

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YEASTS have been reported to occur in media containing high salt or sugar concentrations (LODDER 1932; KROEMER and KRUMBHOLZ 1932; MRAK and BONAR 1932; GRAY 1945; SCARR 1951). Strains resistant to relatively high salt concentrations have also been produced experimentally by several investigators. The adaptation of *Saccharomyces ellipsoideus* to copper sulfate and to sodium chloride has been studied by YANAGISHIMA (1952, 1954), NAGAI (1953), and TAKADA (1953). PERLMAN and O'BRIEN (1954) have reported a strain of *S. cerevisiae* resistant to cobalt nitrate, and SUSSMAN and BRADLEY (1953) have obtained strains of Saccharomyces which tolerate sodium arsenate.

The results reported below arose out of an investigation of the means of achieving and maintaining resistance to relatively high salt concentrations. The questions posed at the outset of the investigation were as follows: Is the effect transitory and if so, is the change which has occurred a cytoplasmic adaptation or an unstable nuclear modification; or, if the effect is lasting, what is the nature of the heritable change responsible for resistance, and what is the pattern of inheritance? Yeast is well-suited to a study of these problems since it will grow in a synthetic medium to which various salts in known concentration may be added. Also, hybrids of known parentage are easily produced and the genetics of resistance may be interpreted from the segregations in asci obtained from the hybrids.

Several genetic strains of yeast (Saccharomyces) have therefore been exposed to media containing deleterious concentrations of potassium chloride, sodium chloride, magnesium chloride, lithium chloride, or manganese chloride, and cultures have been obtained which are resistant to one or another of these salts. In the trials made thus far, it has turned out that only those strains adapted to LiCl or $MnCl_2$ were stable, retaining their resistance after 10 to 20 transfers in normal medium. The first genetic analyses were undertaken with the mutants resistant to LiCl and the results of these are dealt with in this paper.

MATERIALS AND METHODS

The yeast strains for these experiments were provided through the courtesy of MR. D. C. HAWTHORNE. They were heterothallic and morphologically like *Saccharomyces cerevisiae*. Each strain carried a number of genetic markers which were useful as indicators of successful hybridization in the crosses described below.

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The LiCl-free medium in which the cultures were grown consisted of Difco yeast nitrogen base (6.7 g/l), supplemented with adenine and uracil (10 mg/l of each) and glucose (10 g/l). This will be referred to as YNB medium. For a LiCl-containing medium, LiCl was added to the above before autoclaving. Bacto-Agar (2%) was added for solid medium.

The cultures were tested for LiCl resistance as follows: A sample of a culture was inoculated into 5 ml YNB medium and incubated for 24 hrs. The culture was then diluted in sterile tap water and a loopful (ca. 10^4 cells) of the suspension was streaked on plates of solid YNB + LiCl medium. The plates were incubated at 30°C and were examined daily for growth. In describing the experimental results, the term "rapid grower" is applied to those cultures which exhibited after two days as much growth on YNB + LiCl medium as on YNB medium alone; the term "slow grower" describes a culture which made imperceptible or scant growth after two days on the LiCl medium and which exhibited moderate to good growth on the sixth day; the "nongrower" did not show detectable growth during the six-day period of observation.

The cultures were crossed by the mass-mating technique of LINDEGREN and LINDEGREN (1943). A heavy inoculum from each of two cultures of opposite mating type was placed in 2 ml of YNB medium. After zygotes were formed, the cells were transferred to fresh medium at 24 hr intervals for three or four days to enrich the content of diploid cells. Single diploid cells were isolated with a micromanipulator and the cultures derived from these were sporulated on Fowell's medium (FOWELL 1952).

Only asci containing four spores were dissected. All segregants were tested for mating type and for at least four other characteristics, namely, their requirement for adenine, histidine, uracil, and methionine. Only the asci which gave the expected 2:2 segregation ratio for each of the markers are considered in this paper. In the five asci which exhibited unexpected segregation, four were segregating 3:1 for uracil independence:dependence and one was segregating 1:3 for histidine independence:dependence. The reasons for these discrepancies have not been determined.

EXPERIMENTAL RESULTS

Origin of the LiCl-resistant strains

The response of haploid and diploid strains to various concentrations of LiCl in solid YNB medium is indicated by the examples given in table 1. Growth was markedly affected in some strains at a concentration as low as 0.2N; at 0.4N and above, there was no visible growth on the plate two days after plating although there was reasonably good growth of some of these strains after 6 days, at concentrations up to 0.75N. At 1.0N, none of the strains exhibited perceptible growth even after three or four weeks.

In those cases of retarded but definite growth on LiCl medium, the number of colonies which arose on the plate was approximately the same as the number on the control plate; the colonies were simply smaller on LiCl medium. However, individual colonies of distinctly larger size also occurred on LiCl medium. These were especially obvious on plates in which the retardation of general growth was most pronounced

TABLE 1

Growth of haploid and diploid cultures on YNB medium containing various concentrations of LiCl. The resistant mutants 1L.5, 1011L.5, and 1080L.5 arose on plates of YNB + 0.5N LiCl; 1011L1 arose on a plate of YNB + 1.0N LiCl. The amount of growth exhibited after two and six days is expressed as: + = normal or nearly normal; ± = distinctly less than normal; − = imperceptible

Concentration of LiCl	Cultures not previously exposed to LiCl								Mutants selected after exposure to LiCl							
	Haploids						Diploid		Haploids						Diploid	
	1		1011		1073		1080		1L.5		1011L.5		1011L1		1080L.5	
	2	6	2	6	2	6	2	6	2	6	2	6	2	6	2	6
0.0N	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.1	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.2	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.3	−	±	−	+	±	+	±	+	+	+	+	+	+	+	+	+
0.4	−	−	−	+	−	+	−	+	+	+	+	+	+	+	+	+
0.5	−	−	−	+	−	+	−	+	+	+	+	+	+	+	+	+
0.75	−	−	−	±	−	±	−	±	±	+	±	+	+	+	±	+
1.0	−	−	−	−	−	−	−	−	−	±	−	±	+	+	−	±

and they occurred also on plates in which there was no general growth. Samples of such colonies on 0.5N and 1.0N LiCl medium were transferred into YNB medium. After 24 hours these samples were tested for growth on LiCl medium and were found to be resistant to the concentration to which each had been exposed. After 10 to 20 transfers, at 24 hour intervals, in YNB medium, the cultures were again tested on LiCl medium and nearly all were found to have retained their resistance.

Twenty-seven haploid and eight diploid resistant strains were thus obtained. Those isolated from plates containing 0.5N LiCl made as good growth at this concentration after two days as on YNB medium and grew progressively less well on 0.75N and 1.0N LiCl medium (table 1). Those obtained from 1.0N LiCl plates exhibited a normal amount of growth after two days on 1.0N medium.

Inheritance of resistance to 0.5N LiCl

The mutant strain 1L.5 was obtained by plating strain 1 on 0.5N LiCl medium, as described above. Strain 1 itself did not grow at this concentration and grew only poorly, in fact, when exposed to concentrations above 0.2N (table 1). A cross between 1L.5 and 1073, an unadapted strain which exhibited slow growth on 0.5N, yielded the hybrid H23. The hybrid grew as well on 0.5N medium as its resistant parent, 1L.5. Twelve asci were obtained from H23 and the segregations in these asci are given in table 2. Two days after plating, two of the segregants of each ascus had made good growth on 0.5N medium. In three of the asci, the remaining two segregants were slow growers on this medium, exhibiting perceptible growth after four to six days of incubation. In eight of the asci, one of the segregants grew slowly and one did not make perceptible growth in six days. In one ascus, both of the remaining segregants were nongrowers. The presence of two rapid growers in each ascus indicated segregation for a single gene for resistance to 0.5N LiCl and the evidence that the hybrid was also a rapid grower indicated that this gene is dominant. The segregation

TABLE 2

Segregations for resistance to 0.5N LiCl, in asci from hybrid H23

Number of asci	Phenotype of segregants		
	Rapid growth	Slow growth	No growth
8	2	1	1
1	2		2
3	2	2	

TABLE 3

Segregations for resistance to 0.3N and 0.5N LiCl, in asci from hybrid H60. RG = rapid growth; SG = slow growth; NG = no growth

Number of asci	Segregation for growth on					
	0.5N LiCl			0.3N LiCl		
	RG	SG	NG	RG	SG	NG
5	2		2	2	1	1
2	2		2	2		2
1	2		2	2	2	

for slow growth suggested that the hybrid was heterozygous at at least one other locus concerned with resistance to LiCl, the gene or genes for resistance having been contributed by parent 1073, itself a slow grower on 0.5N LiCl medium.

Further evidence that strain 1L.5 carried a single dominant gene for resistance to 0.5N LiCl was obtained from the results of a cross of this strain with one of the nonresistant segregants, 23-9b, from H23. Segregant 23-9b did not show visible growth on medium containing LiCl in concentrations higher than 0.2N. The hybrid from this cross, H60, grew as well on 0.5N medium as 1L.5. Eight asci from this hybrid were dissected and in each of these two of the segregants were resistant to 0.5N LiCl and two were not (table 3).

The conclusion that strain 1073 carried a gene for slow growth in the presence of 0.5N LiCl was tested by crossing 1073 with the nonresistant segregant 23-16d. The hybrid from this cross resembled 1073 in its response to LiCl. In eight asci from H78, two of the segregants were slow growers on 0.5N LiCl and two did not grow.

The cross 1L.5 × 1073 may thus be represented genotypically as $L_x l_y \times l_x L_y$, L_x and L_y being two dominant nonallelic genes for rapid and slow growth, respectively, in response to 0.5N LiCl. Hybrid 23, being $L_x l_x L_y l_y$, would be expected to produce asci of the following three types of segregation: The parental ditype: $L_x l_y$, $L_x l_y$, $l_x L_y$, $l_x L_y$; the nonparental ditype: $L_x L_y$, $L_x L_y$, $l_x l_y$, $l_x l_y$; and the tetratype: $L_x L_y$, $L_x l_y$, $l_x L_y$, $l_x l_y$. The first of these has two rapid and two slow-growing segregants, the second has two rapid growers and two nongrowers, and the third has two rapid growers, one slow grower, and one nongrower. These are the types which were actually obtained from H23 (table 2).

A final test of two-gene segregation in the asci from hybrid H23 was made by crossing the three resistant segregants of a tetratype ascus (23-9) with nonresistant segre-

TABLE 4

Results of crosses of nonresistant strains by three resistant segregants of a tetratype ascus from H23. Segregant 23-9a was a slow grower on 0.5N LiCl; 23-9c and 23-9d were rapid growers

Cross Nonresistant × Resistant	Number of asci	Segregation for resistance to 0.5N LiCl			Genotype of resistant parent
		Rapid growth	Slow growth	No growth	
23-9b × 23-9c	8	2		2	$L_x\, l_y$
23-9b × 23-9d	2	2		2	$L_x\, L_y$
	4	2	1	1	
	1	2	2		
23-16d × 23-9a	8		2	2	$l_x\, L_y$

gants of genotype $l_x l_y$. It was expected that the cross involving the slowgrowing segregant ($l_x L_y$) would yield asci with a 2:2 segregation for slow growth vs. no growth. The crosses involving the rapid growers should produce segregations like those obtained from H23 if the resistant parent were $L_x L_y$, or 2:2 segregations for rapid growth vs. no growth if the parent were $L_x l_y$. These expectations were realized (table 4).

Of the asci in which both L_x and L_y were segregating, five were parental ditypes, two were nonparental ditypes, and twelve were tetratypes. If the two genes were not linked and if only one or neither was linked to the centromere, the asci would be expected in the proportion 1:1:4 (LINDEGREN 1949). Thus, L_x and L_y are either loosely linked or not linked at all.

Resistance to concentrations of LiCl below 0.5N

The results presented above indicated four degrees of resistance to LiCl, exemplified by strains 1L.5, 1073, 1, and the nonresistant segregants of genotype $l_x l_y$. Strain 1, which grew slowly on 0.3N LiCl medium and not on higher concentrations, was crossed with the nonresistant segregant 23-9b, which did not grow at concentrations above 0.2N. The hybrid H77 was like strain 1 in its response to LiCl. In each of the eight asci from this hybrid two of the segregants grew slowly in the presence of 0.3N LiCl and two did not grow at all. Thus strain 1 carries a single dominant gene, L_z, for growth at this concentration.

Since L_x arose in strain 1, it was of interest to determine whether L_x and L_z are at different loci. This was ascertained by retesting on 0.3N LiCl medium the segregants obtained from hybrid H60. If L_x and L_z are nonallelic, the genotypes of the parents of H60 may be presented by $L_x l_y L_z$ (1L.5) × $l_x l_y l_z$ (23-9b), and two-gene segregation would be expected on 0.3N LiCl. The results presented in table 3 indicate that L_x and L_z are at different loci and that there is little if any linkage between them.

As a further test of two-gene segregation, the resistant segregants of the tetratype ascus 60-1 were crossed with nonresistant segregants. Since 60-1a grew slowly on 0.3N LiCl medium and therefore should be $l_x l_y L_z$, the cross 60-1a × 60-2c ($l_x l_y l_z$) was expected to yield 2:2 segregations for slow growth vs. no growth on 0.3N. Segregants 60-1b and 60-1d each grew rapidly on 0.5N LiCl; both therefore should carry L_x and one should have L_z as well. These were crossed with 60-1c ($l_x l_y l_z$), a

TABLE 5

Results of crosses of nonresistant strains by three resistant segregants of a tetratype ascus from H60. Segregant 60-1a grew slowly on 0.3N LiCl and did not grow on 0.5N LiCl; 60-1b and 60-1d grew rapidly on both concentrations. Symbols as in table 3

Cross Nonresistant × resistant	Hybrid number	Number of asci	Segregation for growth on						Genotype of resistant parent
			0.3N LiCl			0.5N LiCl			
			RG	SG	NG	RG	SG	NG	
60-1c × 60-1b	82	3	2		2	2		2	$L_x\ l_y\ L_z$
		3	2	2		2		2	
		3	2	1	1	2		2	
60-1c × 60-1d	83	8	2		2	2		2	$L_x\ l_y\ l_z$
60-2c × 60-1a	84	4		2	2			4	$l_x\ l_y\ L_z$

TABLE 6

Results of cross between 1073 (L_y) and 1 (L_z) to determine whether L_y and L_z are alleles

Number of asci	Segregation for growth on					
	0.3N LiCl			0.5N LiCl		
	RG	SG	NG	RG	SG	NG
6	2	1	1	2		2
2	2	2		2		2

nonresistant segregant from the same ascus. From the cross 60-1b × 60-1c (hybrid H82), nine asci were obtained which exhibited 2:2 segregation when the segregants were tested on 0.5N LiCl medium and two-gene segregations when they were tested on 0.3N LiCl (table 5). Thus 60-1b is $L_x l_y L_z$. The cross 60-1d × 60-1c (hybrid H83) yielded eight asci, in each of which two of the segregants grew on 0.3N and 0.5N LiCl and two did not grow at either of these concentrations. The genotype of 60-1d is therefore $L_x l_y l_z$. In summary, these results confirm the previous evidence that 1L.5 carries both L_x and L_z.

To determine whether L_y and L_z are alleles, 1073 was crossed with strain 1 to produce the hybrid H24. Eight asci were obtained from this hybrid (table 6) and the 2:2 and 3:1 segregations of resistance:nonresistance exhibited in some of these asci when tests were made on 0.3N medium indicates that L_y and L_z are at different loci. Strain 1073 is thus $l_x L_y l_z$.

Resistance to 1.0N LiCl

The resistant mutant 1011L1, which grew rapidly on media containing 1.0N LiCl, was obtained in two steps from the unadapted strain 1011, which is itself capable of slow growth on 0.5N LiCl. A mutant for rapid growth on 0.5N, 1011L.5, which also grew slowly on 1.0N, was first obtained from 1011. The mutant 1011L1 was in turn obtained from 1011L.5 (see table 1 for the range of tolerance of 1011 and the two mutant strains). It should be mentioned that resistance to 1.0N LiCl has also been obtained in a single step from nonresistant strains but has not been analyzed genetically.

TABLE 7

Results of cross between 1011L1, resistant to 1.0N LiCl, and the nonresistant strain 1101-8d

Number of asci	Segregation for growth on					
	0.5N LiCl			1.0N LiCl		
	RG	SG	NG	RG	SG	NG
5	3		1	2	1	1
2	4			2	2	
1	2		2	2		2

A cross was made between 1011L1 and the nonresistant strain 1101-8d to produce hybrid H89, which was like 1011L1 in its response to LiCl. The segregants of eight asci were tested on 1.0N LiCl medium (table 7). Rapid growth was obtained from two segregants of each ascus and, in seven of the eight asci, one or both of the remaining segregants grew slowly. The latter exhibited rapid growth on 0.5N LiCl. Thus it is reasonable to assume that 1011L1 arose in the following way: First a mutation to L_v in the original strain 1011 for rapid growth on 0.5N LiCl medium and slow growth on 1.0N LiCl medium; followed by mutation at another locus to L_w, for rapid growth on 1.0N LiCl medium.

Since L_v and L_z, previously identified in 1L.5, were similar in their response to LiCl, a cross was made between 89-5a ($l_w L_v$) and 60-8a, which was known to carry L_z (table 3). The hybrid H90 grew rapidly in the presence of 0.5N LiCl. In the six asci from this hybrid, segregations of 2:2 (2 asci), 3:1 (3 asci), and 4:0 (1 ascus) were obtained from rapid growth on 0.5N LiCl medium. L_v and L_z are therefore at different loci.

Tests of LiCl-resistant strains on other salt media

To determine whether resistance to LiCl is specific or is a general resistance to salts, several strains of different genotype were plated on media containing potassium chloride, sodium chloride, or manganese chloride. The results are summarized in table 8. All of the strains grew when exposed to KCl in concentrations as high as 2N

TABLE 8

Growth response of various haploid LiCl-resistant and nonresistant strains to media containing additional amounts of KCl, NaCl, and $MnCl_2$. Symbols as in table 1

Strain	LiCl			KCl			NaCl			$MnCl_2$
	0.3N	0.5N	1.0N	1.5N	2.0N	2.5N	1.5N	2.0N	2.5N	0.25N
23-9b	−	−	−	+	+	−	+	−	−	−
1	±	−	−	+	+	−	+	−	−	−
1073	±	±	−	+	+	−	+	−	−	−
60-8a	+	+	−	+	+	−	+	−	−	−
1L.5	+	+	−	+	+	−	+	−	−	−
89-5a	+	+	±	+	+	−	+	−	−	−
89-5b	+	+	+	+	+	−	+	−	−	−
1011L1	+	+	+	+	+	−	+	±	−	−

but not on 2.5N. On NaCl medium, growth was observed on 1.5N but not on 2N, except for 1011L1 (L_vL_w) which grew slowly at the latter concentration. However, there was no indication that the slow growth of this strain was correlated with resistance to LiCl since the two segregants 89-5a (L_vl_w) and 89-5b (l_vL_w) did not exhibit growth on 2N NaCl medium. None of the strains grew on 0.25N $MnCl_2$ medium; this concentration was chosen because mutants resistant to 0.25N and 0.5N have been obtained and will be described elsewhere. In summary, there is no evidence of augmentation in general resistance in those strains selected for resistance to LiCl.

DISCUSSION

The evidence which has been presented indicates that resistance to LiCl is genetically determined, and that several loci are involved. Genes for resistance to relatively low concentrations were present in strains which had not previously been exposed to LiCl; those for resistance to higher concentrations were found in cultures which arose after exposure to the salt. The possibility that LiCl served as a mutagenic as well as a selective agent in these experiments remains to be investigated. It is likely that modifiers affecting resistance were also segregating in the crosses reported above since segregations for slight differences in growth rate on LiCl medium were frequently observed. The degree of resistance of a cell to LiCl depends on the allele for maximum resistance which is present in the cell; i.e., the genes for resistance to LiCl are not cumulative in their effects.

The fact that resistance to LiCl appears to be specific raises the question of whether LiCl exerts a specific effect on the cell or is selectively excluded from the resistant cell by a change in its permeability. The following scattered observations indicate the directions which are being followed in studying this problem. From the results of a spectrophotometric analysis of resistant and nonresistant strains exposed to LiCl, it is unlikely that Li^+ ion penetrates the cell. However, both types of cells, when suspended in LiCl medium for 24–48 hours, lose up to 70 percent of their potassium content, as compared to controls not exposed to salt (LASKOWSKI 1955). Thus, the presence of Li^+ ion in the medium causes a considerable decrease in the K^+ ion concentration normally present in the cell. Several investigators (MUNTZ 1947; SCOTT *et al.* 1950; MEYERHOF and KAPLAN 1951) have shown that the K^+ ion plays an essential role in carbohydrate metabolism in yeast and that neither growth nor fermentation can occur in media lacking this ion. In experiments with K^+-deficient medium (LASKOWSKI 1955), it was found that growth and fermentation were inhibited in LiCl-nonresistant strains but were not inhibited in resistant strains. The K^+-deficient medium was not freed of traces of potassium and it may be that even the resistant strains require small amounts of this ion. These results taken together suggest that the genes for resistance to LiCl may be regarded as genes which suppress the dependence of the cell on potassium, and thus that the selection of resistant mutants is actually a selection for cells with a reduced potassium requirement.

SUMMARY

Several strains of Saccharomyces have been isolated which differ in their resistance to lithium chloride. Mutants resistant to concentrations of 0.5N and 1.0N were ob-

tained by exposure to these concentrations of LiCl. Five genes for resistance have been identified; all of these are dominants and at least three are nonallelic. Resistance to LiCl did not carry with it an augmentation of resistance to other salts, i.e., to potassium chloride, sodium chloride, or manganese chloride. The effect of the Li^+ ion on carbohydrate metabolism and the potassium content of the cells is discussed.

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THE INHERITANCE OF THE DIFFERENCE IN THE COMPOSITION OF THE LIVER MITOCHONDRIA BETWEEN TWO MOUSE STRAINS[1,2]

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IT has been conclusively demonstrated that a large number of enzymes are not freely dissolved in the protoplasm but are concentrated on cytoplasmic particles, especially on the mitochondria (rev. LINDBERG and ERNSTER 1954; SCHNEIDER 1953). Because of their great importance in intracellular metabolism, the behavior of the mitochondria in heredity and their variation under the influence of genes becomes a problem of great interest. This problem can be attacked by demonstrating differences in the properties and composition of cytoplasmic particles from the same organ of two different strains of the same species, and by studying their behavior in crosses. It has been shown (CASPARI and SANTWAY 1954) that liver mitochondria isolated from males of the mouse strains C57 Bl and BALB differ in their phosphorus/nitrogen ratios. It could be shown that at least part of this difference is due to an increased amount of ribose nucleic acid, relative to protein, in C57 as compared to BALB. It was furthermore demonstrated that the character "P/N ratio" is a complex character consisting of at least two components: in the mitochondrial fraction sedimented by low centrifugal force C57 males show a higher P/N ratio than BALB males. In the fraction sedimented by higher centrifugal force the P/N ratio appears to be equal in the two strains. But in the latter fraction, a higher proportion of the total mitochondrial material is contained in C57 males than in BALB males. Since the lighter mitochondria are relatively rich in phosphorus, this fact contributes to the higher P/N ratio of the total mitochondrial preparation obtained from C57 males as compared to BALB.

The present paper constitutes an attempt to describe the behavior of this difference in mitochondrial composition in crosses between the two mouse strains C57 Bl and BALB.

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MATERIALS AND METHODS

The highly inbred mouse strains C57 Bl/subline 11, originally obtained from the Roscoe B. Jackson Memorial Laboratory, and BALB/Ci were used. F_1 and F_2 crosses were carried out in both directions. For the purposes of this paper, F_1 hybrids from crosses of C57 ♀ × BALB ♂ will be designated CB, from crosses

[1] This paper is dedicated to my teacher, PROFESSOR ALFRED KÜHN, on the occasion of his seventieth birthday, as a token of my gratitude and admiration.

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BALB ♀ × C57 ♂ by BC. The CB F_1 was obtained from crosses of 9 C57 females and 2 BALB males, BC F_1 from crosses of 5 BALB females and 2 C57 males. The CB F_2 was derived from 2 CB males and 6 CB females some of which were not sibs. The same applies to BC F_2 for which 3 BC males and 7 BC females were used. In addition, backcross animals from F_1 females from both reciprocal crosses to C57 and BALB males were investigated.

The animals were starved overnight before being used. Liver mitochondria were isolated according to the method of HOGEBOOM, SCHNEIDER and PALLADE (1948), as described previously (CASPARI and SANTWAY 1954). The perfused livers were homogenized in 0.88 M sucrose and, after previous removal of cell debris and nuclei by repeated centrifugation at 600 g, the mitochondria were isolated by centrifugation at 24,000 g for 20 minutes in a Servall SS2 centrifuge. The sediment was washed once in 0.88 M sucrose, suspended in saline and analyzed for nitrogen by the micro-Kjeldahl method and for phosphorus by the Fiske-SubbaRow method. The fluffy sediment described by LAIRD, NYGAARD, RIS and BARTON (1953) was included in the mitochondrial preparations.

It has been shown previously (CASPARI and SANTWAY 1954) that there exists a significant variation in P/N ratios from experiment to experiment. It is therefore of importance to compare animals of different genetic constitutions in the same experiments. In every experiment, eight animals were used since this number could be conveniently handled without involving a long delay between the killing of the animals and the centrifugation. In most experiments, two C57 and two BALB males were used as controls, so that two animals each from two different crosses were compared with each other and with the controls. The ages of the different animals used in the same experiments were approximately equal; no animals under three months of age were used (CASPARI and SANTWAY 1954). In some experiments, four animals each from two different crosses, or two animals each from four different crosses were compared. In some cases, one of the eight samples was lost, either due to accidents during centrifugation, or more frequently because one of the starved animals turned out to be ill or abnormal at dissection. Any animal showing signs of illness or abnormality in any internal organ was excluded from the evaluation. Values obtained in experiments in which one sample was lost, and consequently only one value present for one of the genotypic constitutions, were included in the calculation of means, but were not used in the analyses of variance.

RESULTS

The results obtained from the whole material are summarized in table 1. The table presents the means of the P/N values and their standard errors for each of the strains and crosses. In addition, the table contains an estimate of their variances. As pointed out previously, there exists a significant variability between experiments. Therefore, the values from all experiments involving a particular strain or cross were subjected to an analysis of variance which distinguishes the variance between and within experiments. In all cases, except the two indicated in table 1 by an asterisk, the variance between experiments is significantly, at least at the .05 level, higher than that within experiments. Therefore, in all cases the residual mean square after

TABLE 1

Phosphorus/nitrogen ratios of mitochondria from C57 Bl and BALB males and females and their crosses. In each cross the female parent is designated first; for example, (BC)C means a female from a mating of B female × C male, mated with a C male

Strain	Mean ± SE mg P/gN	n	Residual mean square	df
males				
C57 (C)	102.3 ± 0.70	110	19.98	55
BALB (B)	97.7 ± 0.93	109	40.18	54
CB	97.8 ± 1.33	27	14.89	13
BC	98.7 ± 1.48	27	14.50	13
(CB)(CB)	101.8 ± 0.97	53	27.56	28
(BC)(BC)	98.7 ± 1.00	53	38.52	29
(CB)C	101.1 ± 1.35	40	38.82	24
(CB)B	99.6 ± 1.35	24	24.03	12
(BC)C	102.0 ± 0.97	39	18.17	23
(BC)B	102.2 ± 1.74	24	74.15*	12
females				
C57 (C)	100.3 ± 1.30	37	28.73	19
BALB (B)	97.6 ± 1.16	38	15.11	20
CB	92.5 ± 1.59	24	25.61	16
BC	91.1 ± 1.23	23	9.15	15
(CB)(CB)	91.5 ± 0.86	25	9.21	17
(BC)(BC)	90.1 ± 1.20	25	27.12*	17

* Indicates that in these crosses the mean square between experiments is *not* significantly higher than the residual mean square.

elimination of the variance between experiments is taken as an estimate of the true variance, and indicated in table 1.

Males from the pure strains

The data presented in table 1 show a significantly higher mean P/N ratio for C57 males than for BALB males. This confirms the observation of CASPARI and SANTWAY (1954) on a larger material. The average absolute difference, 4.6 mg P/gN is close to the value found previously, 5.7 mg P/gN. In addition, a difference in residual mean squares between the two strains appears from the table; BALB males show a higher variance between individuals than C57 males. This difference is significant at the .02 level ($F = 2.011$, 54 and 55 df, $F_{.02} \sim 1.90$).

F_1 males

Table 1 shows no difference between the reciprocal F_1's. This is borne out by the analysis of variance which gives no indication for a difference between the two crosses (F for strains/individuals = 1.253, df 1 and 38, $F_{.05} = 4.10$). The material consists of 14 experiments, each of which contained two C57 and two BALB males in addition to two males of each reciprocal F_1. The distribution of the values obtained in this way is given in figure 1. It appears that both F_1's resemble strain BALB. This is borne out by the means of the controls for these experiments which are 99.6 ±

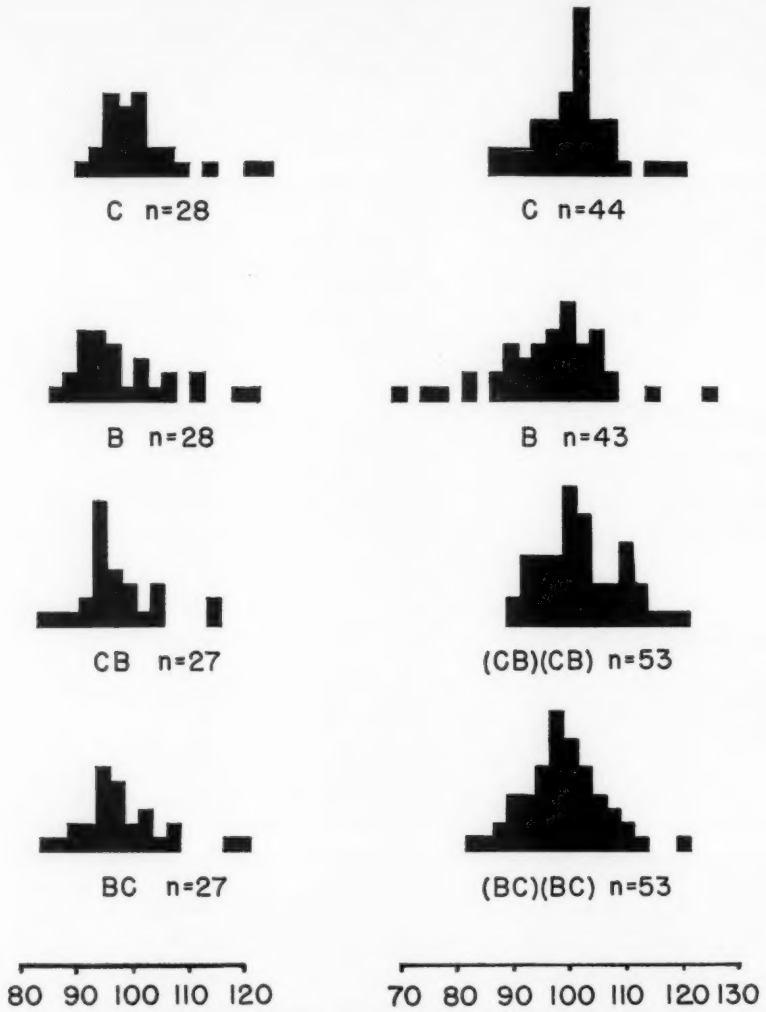

FIGURE 1.—Distribution of values for phosphorus/nitrogen ratios of mitochondria from the livers of F_1 males (left) and F_2 males (right) from reciprocal crosses and their C57 Bl and BALB controls (mg P/gN).

1.72 for BALB and 102.9 ± 1.46 for C57. The difference between the means of both F_1's and C57 is significant at the .05 and .015 level, respectively (t for C57 − BC = 2.014, for C57 − CB = 2.598, for 54 df each).

The residual mean squares of both reciprocal F_1's are lower than those of the in-

TABLE 2

Analysis of variance of phosphorus/nitrogen ratios from reciprocal F_2's

Source of variance	df	Sum of squares	Mean squares
experiments	19	2,374.38	124.97
crosses	1	193.72	193.72
interaction	19	694.35	36.54
individuals	52	1,814.24	34.89
Total	91	5,076.69	

bred strains C57 and BALB. If the two reciprocal F_1's are pooled which is legitimate, because of the lack of a demonstrable difference, an F ratio of 2.728 for 54 and 39 df is obtained for the variances of BALB and the combined F_1's, significant at the .02 level ($F_{.02} \sim 2.08$). The residual mean square of the C57 males is not significantly higher than that of the F_1's.

F_2 males

As shown in table 1, the average P/N ratio of CB F_2 is higher than that of BC F_2. This difference is significant at the 3 percent level ($t = 2.225$, $P < .03$). The same result is obtained by means of analysis of variance, as indicated in table 2. The mean square for the different experiments is, as usual, significantly higher than the residual mean square ($F = 3.538$ for 19 and 71 df, $P < .01$). The variance between strains is significant at the .05 level ($F = 5.484$ for 1 and 71 df, $F_{.05} = 3.98$).

The mean P/N ratios of the control samples for the F_2 are 100.9 ± 1.07 for C57 and 95.6 ± 1.51 for BALB. Comparison with table 1 shows that CB F_2 is very close to C57, and significantly higher than BALB ($t = 3.455$, df = 92, $P < .01$). BC F_2 appears to be intermediate between C57 and BALB; the difference to either one of the control strains is not significant.

The variance of F_2 appears to be increased as compared to F_1. If compared to the combined value for the mean squares of both reciprocal F_1's, CB F_2 has a significantly increased residual variance ($F = 2.615$, 29 and 39 df, $P < .02$). For BC F_2, the difference is in the same direction but not significant ($F = 1.871$, 28 and 39 df, $P < .1$). The distribution of the reciprocal F_2's and their controls is graphically represented in figure 1.

Backcross males

As seen in table 1, no significant difference appears between the means of the backcrosses. In 12 experiments, two males from each of the backcrosses were investigated together. The analysis of variance for these 12 experiments gives no indication for a significant variance due to the different crosses. Further experiments, comparing only (BC ♀ × C57 ♂) with (CB ♀ × C57 ♂) did not give any indication of a difference between these two crosses. In comparison to C57 and BALB in the same experiments, these backcross values appear to be intermediate, but closer to C57. The residual variances, indicated in table 1, are variable. The very high residual mean square observed for males from the cross (BC ♀ × BALB ♂) should be noted.

Influence of color genes

The strains C57 Bl and BALB differ in three major color genes, C57 being *aa BB CC* and BALB *AA bb cc*. In F_2 and the backcrosses, segregation for these genes occurs. An influence of these color genes on the composition of the liver mitochondria would therefore become apparent, if preparations from animals of different color phenotypes isolated in the same experiment are compared. Most of the animals compared were litter mates. The comparison of 27 Agouti and 27 non-Agouti animals showed a difference of 0.548 ± 1.344 mg P/gN in favor of non-Agouti. The average difference for 20 color and albino animals was 0.495 ± 1.886, the average difference for 10 brown and black mice 2.200 ± 4.578. None of these values is significant. There is no evidence that the P/N ratio of the liver mitochondria is affected by the three color genes.

Females

Table 1 shows that the females from strains C57 and BALB differ in their mean P/N ratios in the same direction as the males; but the difference is not significant. However, an analysis of variance for the material from C57 and BALB females, summarized in table 3, indicates a variance due to strains, significant at the .05 level ($F = 6.087$ for 1 and 54 df, $F_{.05} = 4.02$).

There are 12 experiments in which two females from each strain are compared with two males from each strain. The mean P/N ratios of the males and females are not significantly different in either strain. The analysis of variance for these experiments is shown in table 4. There is no indication for a significant interaction, except that the interaction strains × sexes × experiments is close to the .05 level of significance ($F = 1.92$ for 11 and 48 df, $F_{.05} = 1.99$). There is a highly significant variance due to strains which is expected since the males are known to be significantly different, and the females differ in the same direction ($F = 8.20$ for 1 and 71 df, $F_{.01} = 7.01$). The mean square due to sexes is significant at the .05 level ($F = 5.64$ for 1 and 71 df, $F_{.05} = 3.98$).

As shown in table 1, there is no significant difference between the means of the reciprocal crosses in F_1 females. Furthermore, the analysis of variance of the reciprocal F_1's gives no indication for a significant mean square due to the two reciprocal crosses. In six out of eight experiments, the F_1 females were compared with C57 females. The values obtained in these experiments are generally low. (The C57 females used in comparisons with F_1 and F_2 are not included in the mean for C57 females reported

TABLE 3

Analysis of variance for phosphorus/nitrogen ratios from C57 and BALB females

Source of variance	df	Sum of squares	Mean squares
experiments	16	2,762.43	172.65
strains	1	133.66	133.66
interaction	16	337.39	21.09
individuals	38	848.22	22.32
Total	71	4,081.70	

TABLE 4

Analysis of variance for phosphorus/nitrogen ratios from C57 and BALB males and females

Source of variance	df	Sum of squares	Mean squares
experiments	11	1,223.09	111.10
strains	1	190.13	190.13
sexes	1	130.90	130.90
interaction strains/sexes	1	0.71	0.71
interaction strains/expts.	11	133.81	12.16
interaction sexes/expts.	11	281.66	25.61
interaction strains/sexes/expts.	11	541.51	49.23
individuals	48	1,230.33	25.63
Total	95	3,732.14	

in table 1.) The experiments show that the means for both F_1's are slightly lower than those of C57 females (means: BC $F_1 = 91.0 \pm 1.85$, CB $F_1 = 93.7 \pm 2.13$, C57 $= 96.6 \pm 2.06$). This difference is significant at the .05 level for BC ($t = 2.02$, df $= 27$, $P \sim .05$), but not for CB ($t = 0.97$, df $= 28$). Similarly, in F_2 no evidence for a difference between the two reciprocal crosses could be found either by comparison of the means or by analysis of variance. Six experiments in which F_2 females were compared with C57 females indicate that the F_2 means are lower than those of the C57 females (means BC $F_2 = 90.3 \pm 1.73$, CB $F_2 = 89.7 \pm 0.89$, C57 $= 95.4 \pm 2.28$). This difference is significant at the .05 level for CB F_2 ($t = 2.32$, df $= 28$, $P < .05$), not significant for BC F_2 ($t = 1.78$, df $= 28$, $P \sim .07$).

EVALUATION OF RESULTS

The character investigated, P/N ratio of the mitochondria, is a quantitative character. Since it constitutes a ratio of two values, the possibility exists that it may not be normally distributed. The distributions shown in figure 1 and in figure 1 of CASPARI and SANTWAY (1954) appear, however, sufficiently in agreement with a normal distribution to make the statistical treatment legitimate.

There is no doubt that a significant difference exists between the means of the males of the two strains C57 and BALB. The difference is, however, small, and much overlapping between the two strains occurs. In both strains, there is a certain amount of variability inside experiments, and the variance is significantly higher in BALB than in C57. It cannot be decided to what extent the variability inside the strains is due to genetic heterogeneity, since correlations between parents and offspring have not been established because of the significant variance between experiments. In spite of these difficulties, some facts concerning the crosses are well established. The F_1 males have a P/N ratio similar to BALB and significantly lower than C57. There is no evidence for a difference between the males from reciprocal crosses. In the F_2 males, the difference between the reciprocal crosses is significant at the .03 level and is in the direction of the grandmaternal strains. There appears to be no difference between the males obtained from backcrosses of F_1 females from the two reciprocal crosses to males from the pure strains. All four backcrosses have P/N ratios which appear to approach the C57 controls.

The similarity of F_1 males to BALB may be interpreted to mean that heterozygotes for the genes determining P/N ratio of the mitochondria show the BALB phenotype, independently of the X chromosome, of the cytoplasm and of the Y chromosome. The difference between males from reciprocal crosses found in F_2 must be due either to the cytoplasm or to the Y chromosome, since the reciprocal F_1's must be assumed to be identical with respect to their autosomes and their X chromosomes. Since this difference between reciprocal crosses is not found in F_1, it must be concluded that its appearance in F_2 is due to the establishment of homozygotes. In other words, the high P/N ratio in CB F_2 appears to be due to a cooperation between genes in homozygous condition and the cytoplasm or the Y chromosome.

Whether the factor collaborating with the genes is the cytoplasm or the Y chromosome cannot be decided with certainty. It seems preferable to regard the cytoplasm as responsible since CB F_2 has the Y chromosome from BALB, and it would have to be concluded that the action of the Y chromosomes, as seen in F_2, is opposite to the direction of the difference between the pure strains. In addition, similar cases of interaction of plasmon-sensitive genes with certain cytoplasms have been frequently described in the literature (CASPARI 1948). Dependence on single recessive genes which are expressed only in a specific cytoplasm has been described for pollen-sterility in Linum (BATESON and GAIRDNER 1921), for a flower abnormality in Epilobium (Michaelis 1940), for sex determination in Streptocarpus (OEHLKERS 1941). In analogy to these cases, the genetic behavior of the mitochondria may be interpreted as being influenced by genes which in homozygous condition increase the P/N ratio in C57 cytoplasm but not in BALB cytoplasm. The heterozygotes are not plasmon-sensitive. Segregation of genes affecting mitochondrial P/N ratio is suggested by the fact that both in F_2 and in the backcrosses the variances inside experments are increased as compared to F_1.

The mean P/N ratio of CB F_2 males is indistinguishable from C57. This is astonishing since in F_2 many genes should be present in heterozygous condition, and induce a P/N ratio similar to BALB. As an interpretation, it may be suggested that plasmon-sensitive genes derived from BALB may also induce an increased P/N ratio in C57 cytoplasm when present in homozygous condition. This assumption seems to agree with the finding of MICHAELIS (1942) that genes derived both from the maternal and from the paternal side may appear plasmon-sensitive in crosses, and that in addition their plasmon sensitivity depends on the remainder of the genotype. The assumption that genes derived from BALB in homozygous condition may induce a higher P/N ratio in C57 cytoplasm may also explain the high value obtained in the backcross CB ♀ × BALB ♂. The mean from the backcross BC ♀ × BALB ♂ may be regarded as unreliable because of its high standard error. The high variance inside experiments in this cross recalls the original BALB.

Since the character investigated is a quantitative character, polygenic inheritance may be expected to occur. This is also suggested by the previous finding that the character "P/N ratio" is actually a composite character in which at least two different components are involved. It is, however, impossible on the basis of the data presented to estimate the number of genes involved.

The P/N ratios of the mitochondria from the females of the two strains differ from each other in the same direction as the males. But the difference is quantitatively

smaller and statistically insignificant. Evidence for a genetic basis of the mitochondrial constitution in the females is derived from the fact that both F_1 and F_2 females have lower P/N ratios than C57 females. Of the four comparisons possible, two are significant at the 5 percent level and one more at the 7 percent level; it may be concluded that at least some of these differences are real. The lack of a difference between the pure strains and between the reciprocal F_2's in the females could suggest that the difference in the F_2 males may be due to an influence of the Y chromosomes; but this assumption appears to be unlikely in view of the fact that Y chromosomes would be assumed to act in an opposite direction in the pure strains and in F_2. It appears preferable to assume that the difference in mitochondrial composition between males and females is sex-controlled. This interpretation agrees with the evidence that mitochondria may be affected by hormones. Hexokinase activity, e.g., is known to be influenced by a variety of hormones, including estrogen, and has been shown to be largely restricted to the mitochondrial fraction (CRANE and SOLS 1953).

DISCUSSION

On the basis of the data reported, the mitochondrial P/N ratio appears to be dependent on segregating genes in addition to a cytoplasmic component. This interpretation is in agreement with the genetic behavior of mitochondria in yeast, as analyzed by EPHRUSSI and collaborators (rev. EPHRUSSI 1953). In yeast, differences in mitochondrial activity have been shown to depend on both nuclear genes and cytoplasmic factors.

The manner of interaction between the genes and the cytoplasmic component involved recalls the interaction of genes and plastids in Oenothera, as described by RENNER (1936) and SCHWEMMLE *et al.* (1938). In these cases, a certain plastid constitution is transmitted independently of the genes. This differential plastid constitution is not expressed directly in a phenotypic difference, but in the differential reaction of the chloroplasts to certain genotypic constitutions.

The question arises whether the cytoplasmic factors responsible for the reaction of the mitochondria on chromosomal genes are the maternally transmitted mitochondria themselves. The question whether or not the mitochondria constitute self-reproducing entities has been frequently discussed. The fact that cells without microscopically visible mitochondria have been found to be viable and able to produce visible mitochondria does not exclude the possibility that microscopically invisible stages of mitochondria exist, as has indeed been suggested on the basis of electron-microscopic observations (EICHENBERGER 1953). It has been pointed out by SONNEborn (1950) that the only valid criterion of self-reproduction consists in the occurrence of independent mutations and the transmission of the mutated state to successive generations independently of genotype and environment. This criterion has been met for mitochondria in EPHRUSSI's experiments in yeast and for the mitochondrial elements in a melanotic mouse tumor (WOODS, DUBUY, BURK and HESSELBACH 1949). The observations reported in the present paper point in the same direction. However, caution is indicated because the independent transmission of the difference has been demonstrated for only two generations, and the possibility that the independently transmitted factor may be the Y chromosome is not excluded.

It appears from the data that the variance of mitochondrial P/N ratio in F_1 is

reduced as compared to at least one of the parental strains. A decrease in variance in F_1 has been repeatedly found in experiments involving quantitative characters, e.g., by GRÜNEBERG (1950) in the mouse, by ROBERTSON and REEVE (1952) and by DOBZHANSKY and WALLACE (1953) in Drosophila, and by MATHER (1950) in Primula. This phenomenon is an expression of genetic homeostasis (LERNER 1954; DOBZHANSKY and WALLACE 1953) and is regarded as one of the main factors responsible for the superiority of heterozygotes. It is interpreted to mean that in heterozygous organisms the developmental processes are better buffered than in homozygotes (LERNER 1954; GRÜNEBERG 1954). Superior homeostasis in the heterozygotes with respect to mitochondrial constitution would supply a conceivable physiological mechanism for this phenomenon because of the important function of the mitochondria in cellular metabolism. It has been suggested by LINDBERG and ERNSTER (1954) that one of the main functions of the mitochondria consists in controlling the rate of oxidative breakdown and consequently the availability of substrates for synthetic processes within the cell. Under this assumption, the mitochondria themselves would be important mechanisms in maintaining the homeostasis at the intracellular level. Genetic control of the homeostasis of mitochondrial constitution and function could well represent the physiological basis for the superiority of certain genotypes.

SUMMARY

1. The P/N ratios of isolated liver mitochondria from the mouse strains C57 Bl and BALB and their crosses have been determined.

2. It is confirmed that C57 Bl males show a significantly higher P/N ratio than BALB males.

3. F_1 males have a mitochondrial P/N ratio similar to BALB and significantly lower than C57. No reciprocal differences are found in the F_1 males.

4. The mitochondrial P/N ratios of F_2 males show a difference between reciprocal crosses significant at the 3 percent level. The variance in F_2 is increased over F_1, indicating genic segregation.

5. Males from backcrosses of F_1 females from both reciprocal crosses to males from the original strains do not show any demonstrable differences in P/N ratio.

6. The data are interpreted in terms of interaction of a cytoplasmic factor with plasmon-sensitive genes. A high mitochondrial P/N ratio results from the presence of genes in homozygous condition in the cytoplasm derived from C57. The possibility that the Y chromosome is involved in the determination of the mitochondrial P/N ratio is not excluded.

7. No evidence for an effect of the color genes agouti, black and albino on mitochondrial constitution has been observed.

8. C57 and BALB females differ from each other in the same direction as the males, but not significantly so. Evidence for genetic control of mitochondrial P/N ratio in females is obtained from the fact that in F_1 and F_2 consistently lower values are found than in C57 control females.

9. The variance between individuals is significantly larger in BALB than in C57. The variance in F_1 appears to be reduced. It is suggested that this finding may indicate an increased homeostasis of mitochondrial constitution in the heterozygote,

and that this phenomenon may provide a physiological basis for heterozygote superiority.

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A HIGHLY SPECIFIC COMPLEMENTARY LETHAL SYSTEM IN DROSOPHILA MELANOGASTER

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IN January, 1954, two prune-3[1] females (*Drosophila melanogaster*) were mated to three males from the *S/E-S* stock. There resulted 339 females (all, as expected, pn^+), and one bobbed male that was also pn^+ (it was later found that the *S/E-S* stock carried *bb*, so this male was clearly a nondisjunctional exception). The absence of the class of pn^3 males was wholly unexpected, since the usual result would be that such males would be as numerous as their pn^+ sisters. Several possible interpretations were tested, but the only one that turned out to be adequate was that there is a dominant autosomal gene (now called "Prune-killer", symbol *K-pn*) in the *S/E-S* stock, that kills all prune flies but is without effect on pn^+. Later experiments have shown that the *S/E-S* stock is homozygous for *K-pn*; rather extensive tests have failed to show its presence in any other stock—both mutant stocks and wild strains from widely scattered localities having been tested. It has also been found that *K-pn* is equally effective in killing pn^1, pn^2, or pn^3—the only independently arisen alleles at present available for study—and that *pn* females are killed as effectively as are *pn* males. No other effects of *K-pn* have been found. It has no detected effect on the phenotype in any combination tried; in particular, it has no modifying effect on the color of the eyes in any of the numerous mutant eye-colors with which it has been tried.

The experiments leading to these and other conclusions may now be outlined.

GENETIC TESTS

Seventeen females from the original culture were tested. One of them was shown, by further testing, to carry an ordinary sex-linked lethal. Of the others, 8 were mated to *pn* males: they produced 745 + ♀♀, 393 *pn* ♀♀, 723 + ♂♂, 351 *pn* ♂♂—i.e., about 2 + :1 *pn* among both sons and daughters. Seven females were mated to males from the *S/E-S* stock, and produced (disregarding S): 756 + ♀♀, 414 + ♂♂—i.e., the expected *pn* sons were absent. The remaining female was mated to an unrelated + male, and produced: 125 + ♀♀, 60 + ♂♂, 34 *pn* ♂♂—i.e., half her *pn* sons were missing.

These results were sufficient to indicate that a dominant autosomal gene was involved; it was also apparent that this gene could not be closely linked to *S*, since in the above tests some of the females used were *S*/+, and there was no obvious deviation from the expected proportions for *S* and S^+ among their offspring. However, *S* is near one end of the second chromosome, and is therefore not an efficient marker for that chromosome in heterozygous females. Accordingly a test was made that depended on the absence of crossing over in male Drosophila.

[1] The names of mutants and the symbols for the genes concerned are the standard ones used in Drosophila. For descriptions, loci, etc. see BRIDGES-BREHME 1944.

The uniformity of the *S/E-S* stock was first tested. Twelve matings (including the original one that led to the present study) were made of the type *pn*^{3} ♀ × *S/E-S* ♂. There resulted 1629 + ♀♀, 0 *pn* ♂♂, 4 *bb* ♂♂ (nondisjunctional and XO). Two matings of *pn*^{1} ♀ × *S/E-S* ♂ gave 198 + ♀♀, 0 ♂♂; one *pn*^{2} ♀ × *S/E-S* ♂ gave 121 + ♀♀, 0 ♂♂; one *pn*^{1}/*pn*^{3} ♀ × *S/E-S* ♂ gave 198 + ♀♀, 0 ♂♂. These tests, plus those listed above from matings to F_1 females, show that at least 23 males (probably more, since more than one male was used in most cultures) from the *S/E-S* stock were unable to produce *pn* offspring. It has therefore been assumed that this stock was homozygous for the gene concerned—and all experiments designed on this assumption have given results consistent with it.

The *S/E-S* stock was mated to males carrying the dominants *Pin* (chromosome II) and *Ubx* (chromosome III—formerly called *bx*^{D}), and F_1 *Pin*; *Ubx* males were mated to *pn*^{3} females. There resulted 227 *pn*$^+$ ♀♀, 92 *pn* ♂♂—i.e., half of the *pn* offspring were absent. Further, the 92 *pn* males were classified for the dominant markers: there were 50 *Pin Ubx*, 42 *Ubx*. *K-pn* showed no recombination with *Ubx*, and is therefore in the third chromosome.

The determination of the locus of *K-pn* in the third chromosome has been somewhat more troublesome. The first test was with the two dominants Lyra (*Ly*, locus 40.5) and Stubble (*Sb*, locus 58.2). Males *pn*^{3}; *Ly Sb*/+ were mated to females from the *S/E-S* stock. The resulting females of the constitution +/*pn*^{3}; *S*/+; *Ly Sb*/*K-pn* were mated to *pn*^{3} males. There resulted (disregarding *S*) 525 *pn*$^+$, and the following 252 *pn*: *Ly Sb* 117, *Ly* 18, *Sb* 24, +93. These data show only that *K-pn* is not between *Ly* and *Sb*, nor is it near either one—i.e., it is near one end or the other of the chromosome.

This result showed that it would be necessary to use multiple recessive stocks to locate *K-pn*, and for this reason it was desirable to establish a strain of it free of *S* (which interferes with the classification of some third chromosome markers) and of *E-S* (which carries an inversion in II that might affect crossing over in III). Accordingly, from the test just described, *pn*$^+$; *Sb* males were selected and tested individually by *pn*$^+$/*pn* females. One such male gave no *pn*; *Sb* sons. His *pn*$^+$; *Sb* sons were mated to females from the *S/E-S* stock, and the *S*; *Sb* offspring were mated together. These flies were of the composition *S*/+; *Sb K-pn*/ *K-pn*. From their offspring *S*$^+$ were selected, and a stock maintained that has in fact been shown to be homozygous for *K-pn*. *Sb* has been maintained in it by selection, since for some experiments it is useful to have the *K-pn* chromosome marked by a dominant.

Two methods have been used to locate *K-pn*. One is to mate *Sb K-pn*/ *K-pn* to a multiple recessive, mate the F_1 *Sb* females to the same multiple recessive, and test various types of *Sb*-carrying crossover sons individually by *pn* females. In case the tested crossover carries *K-pn*, no *pn*; *Sb* sons are produced, whereas if the crossover does not carry *K-pn* the *pn* sons are *Sb* and *Sb*$^+$ in equal numbers. Tests of this sort soon showed that *K-pn* lies near the right end of the chromosome, and accordingly the second method was used to get a more precise location.

This method is like that used in the *Ly Sb* test described above, and gives a direct testcross for *K-pn*; but it requires the production of somewhat more complex stocks. Several such tests were made; two of them may be recorded here, since the others gave no additional essential information.

Females of the composition $\frac{+}{pn}$; $\frac{Sb \quad ca \; K\text{-}pn}{e^s}$ were mated to e^s *ca* males. The *pn* offspring were of the following classes (numbers in parentheses indicate the intervals in which crossing over occurred): (0) e^s, 88; (1) *Sb* e^s, 14: (2) *Sb*, 38; (3) *Sb ca*, 4; (1, 2) +, 2; total, 146. It follows that *K-pn* lies to the right of *ca*. There is only one marker gene now available that lies further to the right than *ca*—namely, brevis (*bv*). Accordingly, two tests were carried out: (A) $\frac{+}{pn}$; $\frac{ca \quad K\text{-}pn}{bv}$ and (B) $\frac{+}{pn}$; $\frac{\quad K\text{-}pn}{ca \; bv}$, both kinds of females being mated to *ca bv* males—which in most cases were also *pn*. The following results were obtained:

	pn⁺ offspring		*pn* offspring			
	0	1	0	1	2	1, 2
A	971	25	204 (*bv*)	3 (*ca bv*)	0 (*ca*)	0 (+)
B	620	8	443 (*ca bv*)	9 (*bv*)	1 (+)	0 (*ca*)

(The relative numbers of *pn*⁺ and *pn* here are not significant, since in some cultures the fathers were *pn*⁺ and in others they were *pn*—and also the *pn*⁺ were not classified for *ca* and *bv* in all cultures.)

These data give the locus of *K-pn* as 0.2 units to the right of *bv*. The latter is listed by BRIDGES-BREHME (1944) at 104.3, with only one locus—*M(3)g*—to the right of it, at 106.2. This latter mutant has been lost, and in view of its slight phenotypic effect, dependence on a linked enhancer, and phenotypic resemblance to brevis, one may question the accuracy of the determination of its locus. Brevis is listed as 3.6 units from *ca*; the present data give $100 \times 45/2284 = 2.0$ for the recombination percentage. In view of BRIDGES' custom of using maximum observed recombination values for map distances, this is a reasonable agreement with expectation, and the locus of *K-pn* may be put at 104.5, making it the rightmost locus now available for study in the third chromosome.

DR. E. B. LEWIS has kindly examined the salivary gland chromosomes of *K-pn* larvae; he finds the right end of chromosome III normal in appearance.

Various kinds of tests other than those already described have been carried out, and have confirmed the conclusions there drawn. Attached-X *pn* females × *K-pn*/*K-pn* males gave *pn*⁺ sons and a few *pn*⁺ superfemales; attached-X *pn*⁺/*pn*⁺; *K-pn*/*K-pn* females × *pn* males gave *pn*⁺ daughters and no sons. The first of these two results shows that *pn*/*pn*/*pn*⁺; *K-pn*/+ survives—a conclusion confirmed by the use of two different short duplicating fragments containing *pn*⁺. In this way it is possible to obtain *pn*/*pn*⁺; *K-pn*/+ males, and *pn*/*pn*/*pn*⁺; *K-pn*/+ females, that are viable and fertile, since the *pn*⁺ is contained in a small duplicating fragment that does not seriously affect viability or fertility. (The duplications used were *Dp* (*1;f*) X^{c2} and *Dp* (*1;4*)^{m5}—see BRIDGES-BREHME 1944).

Properties of K-pn

From the mating of *pn/pn* ♀♀ × *K-pn/K-pn* ♂♂, all the regular daughters are viable and all the regular sons die. Examination of such cultures, when the parents are transferred daily, shows that the eggs hatch and the larvae appear normal. However, the male larvae (*pn*; *K-pn*) do not develop beyond the second instar. They appear superficially normal until the time when their sisters molt at the end of the second instar; at this time they usually crawl out of the food, and all of them die without undergoing the molt. No detailed study of them has been made, but no structural abnormality has been observed.

It is clear from some of the tests described above that *pn* females carrying *K-pn*, and *pn* males that are homozygous for *K-pn*, also die; no attempt has been made to determine the time of death for these classes.

It was hoped that mosaics might be obtained that would contain parts that were *pn*; *K-pn* in composition, in order to determine the phenotype of specific regions of that composition. Two different tests were carried out, using *y pn* so that yellow bristles and hairs would serve as an index of the presence of *pn*, and using ca^{nd} (LEWIS and GENCARELLA 1952) as a source of mosaicism. It does not seem necessary to present the details of these experiments, since they were not wholly conclusive. There was a deficiency of mosaics carrying *K-pn* and with male parts *pn* in composition—but it is not certain that this deficiency was statistically significant. All such mosaics obtained had the yellow (i.e., male and genetically *pn*) parts confined to the thorax and abdomen—but again it is not certain (though it is probable) that the absence of *pn* eye-color was significant. In any case, the experiments did not answer the question of the possible effect of *K-pn* on eye color.

There is one reason for suspecting that *K-pn* may be related to pigment formation: the *pn*; *K-pn* larvae, that die at the end of the second instar, have been observed to have slightly paler Malpighian tubes than control *pn* larvae at the same age. However, it may well be that this is a symptom of approaching death, rather than a specific effect of *K-pn* on pigment synthesis.

K-pn/+ has been combined with a wide variety of eye-colors other than *pn*, and in no case has it been found to have any modifying effect on them. The list tried includes, among others: *v*, *cn*, *st* (which remove the brown pigment); *bw* (which removes the red pigment); w^a, w^e, zeste (which are known to be very susceptible to modifiers); *ca* (which lies near *K-pn*); and chocolate, *se*, and *pd* (which somewhat resemble *pn*). In the latter case, BRIDGES-BREHME (1944) described a special modifier of *pd* (purpleoid), called Purpleoider (*Pdr*) which has effects on *pd* somewhat similar to the effects of *K-pn* on *pn*. Tests have shown that *K-pn* does not affect *pd*, and that *Pdr* does not affect *pn*.

If *K-pn* kills *pn* through an action on some substance present in *pn* but not in other flies, it seemed possible that this hypothetical substance might be involved in the production of eye-pigment, and that it might be absent in white-eyed flies. Accordingly *pn w*, and *pn*; *cn bw* (both with eyes without pigment) were tried; *K-pn* was fully effective in killing both types. Such a result was perhaps to have been expected, since the *pn*; *K-pn* larvae die at a stage where the synthesis of eye-

pigment has presumably not yet begun—although the pigment of the Malpighian tubes (also absent in *w* and in *cn bw*) is already present in *pn* larvae at that stage.

DISCUSSION

The case of *pd* and *Pdr*, briefly referred to above, is somewhat like the present one. It differs in that each mutant gene has an effect on the color of the eyes in the absence of the other one, and in that the lethal effect is found only when both genes are homozygous (BRIDGES-BREHME 1944). No detailed account has ever been published.

Another analogous situation is that found with crossveinless (*cv*) and fused (*fu*) in *Drosophila simulans* (STURTEVANT 1929). Fused has reduced viability, and this is greatly exaggerated in *cv fu*, which has a phenotype that appears to be the simple sum of *cv* and *fu*, but has such a low viability that it rarely survives to the adult condition.

Perhaps a closer analogy is provided by the "synthetic lethals" described for *D. pseudoobscura* by DOBZHANSKY (1946). In this case it was found that two second chromosomes, neither of which had a lethal effect, might produce a lethal chromosome by crossing over—presumably through the bringing together of complementary recessive genes.

The situation is also similar to the well-known examples of genes that are lethal or cause tumors in species hybrids but not in the species in which they are normally found. (See, for example, HOLLINGSHEAD 1930 and GORDON 1931.)

There are other more or less similar examples scattered through the literature—cases where genes, that themselves may or may not have observed phenotypic effects, produce unexpectedly great effects on phenotypes conditioned by other genes. One has only to refer to the extensive literature on "enhancers" or "specific intensifiers" on the one hand, or "suppressors" on the other hand, to locate numerous examples.

K-pn may be thought of as an extreme "enhancer"—though it is not known whether it has any effect on the most obvious phenotypic effect of *pn* (the eye color); and if *pn* has any effect on viability (as it presumably does), it is not one that is easily detected in ordinary experiments.

It may be that specific "killers" are more frequent than would be inferred from the previous failure to recover any as effective and as specific as *K-pn*. This will become evident if it is recognized that the present case owes its discovery to the facts that *pn* is a sex-linked recessive and that *K-pn* is an autosomal dominant. The analysis would probably have failed if it had not happened that the *S/E-S* stock was homozygous for *K-pn*. It seems probable that these rather special requirements for discovery and analysis will not be met with in many cases—i.e., it is probable that recessive "killers" exist, that autosomal mutants are subject to such killing, and that most "killers" exist in heterozygous form in the stocks where they occur.

There is one use to which the present system may be put, in the design of experiments. In the two matings

pn ♀ × *K-pn*/ *K-pn* ♂

attached-X *K-pn*/ *K-pn* ♀ × *pn* ♂

no males are produced except by nondisjunction (in the first case) or detachment of attached-X's (in the second case). These two matings therefore give convenient and efficient methods of producing large numbers of all-female cultures—and therefore of necessarily virgin females. For some types of experiments the procedure may be found useful.

One possible hypothesis is that *pn* leads to the production (or accumulation) of some substance that is absent, or present only in small amounts, in pn^+ flies, and that *K-pn* converts this substance to a different and toxic substance. Such a hypothesis at least suggests experiments to be carried out, and offers some hope of relating the phenomena to the reactions concerned in the synthesis of pigments.

SUMMARY

1. The *S/E-S* stock was found to be homozygous for a gene called "Prune-killer" (symbol, *K-pn*), that is located at 104.5 in chromosome III.

2. *K-pn* has only one known effect: it causes the death of all prune flies that carry it, this effect being dominant.

3. This lethal effect of *pn*; *K-pn* occurs with pn^1, pn^2, or pn^3—the only separately arisen *pn* mutants available.

4. In the only case studied—males that are *pn*; *K-pn*—death occurs at the end of the second larval instar, and no gross abnormality was detected in the dying larvae.

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RELATION OF UNEQUAL CROSSING OVER TO THE INTERDEPENDENCE OF R^r ELEMENTS (P) AND (S)[1, 2, 3]

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PREVIOUS studies have shown that the gene R^r behaves, with respect to spontaneous mutation, as if it were composed of two independently mutating elements: (P), affecting anthocyanin pigmentation of specific plant tissues, and (S), affecting anthocyanin pigmentation of the aleurone layer of the endosperm (STADLER 1948). When seed-color is lost by mutation, the level of plant-color expression remains unaltered. Similarly, the mutation of the plant-color determiner has no effect on the phenotypic expression of seed-color (STADLER 1951a).

Recent experiments have shown that (P) and (S) are separable by unequal crossing over (STADLER and NUFFER 1953). It should be noted that the symbols (P) and (S) are not intended to imply that there is only one separable element for plant-color and one for seed-color. Both could be multiple (PPPSSSS) and give the same experimental results. These symbols indicate simply that all (P) determiners may be removed in a proximal segment that leave some (S), and that all (S) elements may be removed in a distal segment that will leave some (P).

Thus the gene R^r may be regarded in either of two ways. The R^r effect may be produced by the action of two separate genes of independent action, one affecting the aleurone-color and the other affecting the plant-color, or alternatively, it may be thought of as a single or compound structure with dual gene action.

These possibilities may be represented in two models:

A	B
Independent genes	*Single gene*
P—S	R^{PS}
↓	↓
p—S	R^{pS}

The experimental distinction between these two models is in the structure of the gene following mutation. According to model A, the mutation of element (P) would have no effect on the subsequent mutation frequency of element (S), since (S)

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[2] An abstract of this paper has been published in Science **119:** 585, April, 1954.

[3] Contribution from Missouri Agricultural Experiment Station, Journal Series No. 1541. Approved by the Director.

[4] Deceased May 12, 1954. This manuscript was prepared after the death of the senior investigator, but the major part of the research was conducted prior to his death. The co-investigator assumes responsibility for the contents of this paper.

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would be the same gene before and after the mutation of (P). With the model B, however, the mutation frequency of (S) cannot be predicted, since it would depend upon the mutability of the new gene designated R^{pS}.

When several mutant R^g alleles (designated pS) were compared with their R^r alleles (designated PS), it turned out that the frequency of aleurone-color mutation in the parent R^r alleles was approximately 10 times as high as that in the mutant R^g alleles (STADLER 1946 and 1951b). The results are those that would be expected with model B, a single or compound gene.

These facts taken together seem to indicate that while (P) and (S) are two distinct structural elements, they still have special inter-relationships as members of a functional complex.

The present paper is concerned with the behavior of one particular R^g Cornell allele, designated R^g-14, in subsequent mutational trials (STADLER and EMMERLING 1954). Briefly, the R^g-14 allele exhibits two peculiarities in its mutational behavior. (1) In the heterozygote R^g-14/R^r the R^g gametes mutate at a lower rate than those of the R^r gametes. (2) In the backcross progeny the R^r gametes in R^g/R^r plants mutate at a higher rate than those of the R^r gametes in the homozygous sibs. The latter phenomenon will be referred to as the M_G effect.

Considering, then, these two characteristics of R^g-14, three alternative hypotheses can be formulated to account for the observations.

(A). The reduction in the mutation frequency of R^g-14 is due wholly to a change in the stability of (S) following either mutation or loss of (P). The M_G effect is unexpected on this basis unless it can be attributed to a modifier of mutation rate. However, extensive trials have failed to separate the M_G effect from the R^g-14 gene. Thus the effect must be either closely linked or associated with the R^g-14 allele. In this connection it might be noted that an *R*-linked modifier, which depresses the frequency of seed-color mutation, has been identified previously in the R^g McBain stock (STADLER 1950).

(B). The genic element (S) does not change in stability, but both effects are due entirely to unequal crossing over, yielding r^r crossovers in addition to r^r mutants from noncrossover mutation of R^r.

(C). The stability of (S) is affected by mutation or loss of (P), and the occurrence of unequal crossing over increases the number of r^r mutants from R^g-14/R^r, thus giving the M_G effect.

EXPERIMENTAL EVIDENCE

Preliminary data on the compound *G* R^g-14 *k*/*g* R^r Cornell *K* support the view that unequal crossing over is involved in the production of some of the seed-color mutations.

The aleurone-color mutations were secured from the cross *G* R^g-14 *k*/*g* R^r Cornell *K* × *g* r^g *k*/*g* r^g *k*. The stock carried markers on either side of the *R* locus which permits the detection of unequal crossovers. The proximal marker used was golden plant (designated *g*), located 14 units to the left of *R*. Since no gene distal to *R* is known, it was necessary to use a cytological marker. A large heterochromatic knob located one unit to the right of *R* (RHOADES 1942) was used. This knob (designated

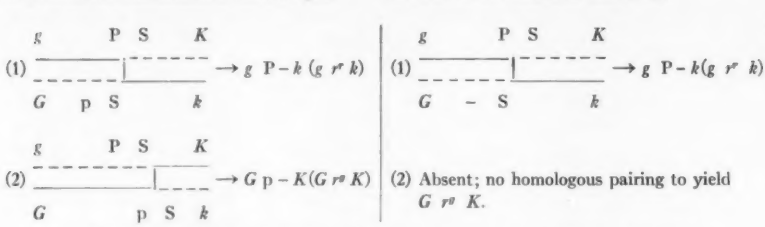
A If R^g-14 is a noncrossover mutant (pS).
B If R^g-14 is a crossover case (–S).
g P S K
(1) → g P – k (g r^r k)
G p S k
g P S K
(2) → G p – K(G r^g K)
G p S k
g P S K
(1) → g P – k(g r^r k)
G – S k
(2) Absent; no homologous pairing to yield G r^g K.

FIGURE 1.—The types of unequal crossovers expected by loss of seed-color. *G* R^g-14 *k*/*g* R^r: Cornell *K*

K) is known to segregate preferentially at megasporogenesis (RHOADES 1942). In the progeny of females heterozygous for knob 10, individuals carrying the knob are recovered about three times as frequently as those without the knob.

The *G* R^g-14 *k*/*g* R^r *K* plants were pollinated by *g* r^g *k* pollen in a detasseled plot planting, and the aleurone mutations were identified by inspection of all seeds on the ears. The seed-color mutants were grown to maturity to determine the genotype and were pollinated by a recessive *r* stock to verify the mutation. Each mutant was sampled cytologically to determine whether unequal crossing over had occurred.

The classes of unequal crossovers expected by loss of seed-color are illustrated in figure 1. The classification of unequal crossovers indicated for the R^g-14/R^r compound is derived from the assumption that the R^g-14 allele may be either (pS) or (–S) in constitution. Presumably the R^g-14 allele of the (pS) type represents a noncrossover, plant-color mutation bearing a mutant null allele of (P) which has lost its phenotypic action but has retained its pairing affinity. Thus the compound *G* R^g-14 *k*/*g* R^r *K*, including the (pS) allele, should yield the apparent mutants r^r *k* and r^g *K*, since both distal and proximal displacement of the knob-bearing segment may occur (see fig. 1A). However, it is possible that R^g-14 is deficient for the element (P) because of the result of a plant-color, unequal crossover in the parent R^r allele. In this case the compound *G* R^g-14 *k*/*g* R^r *K*, including the (–S) allele, should produce r^r *k* unequal crossovers, with none of the alternative class (see fig. 1B). This is based on the assumption that unequal crossing over occurs only when there are two or more synaptically homologous elements present in the gene string. Thus the critical crossover class, the r^g *K* type, is expected from compounds of R^r with the R^g-14 allele of (pS) constitution and not from the (–S) type.

But the absence of the critical crossover class would not be a valid criterion for distinguishing between (–S) and (pS) alleles if phenotypically inactive elements were present either distal to (S), or proximal to (P), or between (S) and (P). These null elements (designated N) could also be capable of pairing with (S) or (P) and thus produce unequal crossovers of the type expected only from the (pS) alleles.

Experimental detection of N elements either proximal to (P) or distal to (S) would come from the homozygote R^r Cornell (PS)/(PS), cytologically marked for the detection of unequal crossing over. If R^r Cornell consists of (NPS) or (PSN), unequal crossing over could yield r^g crossovers as well as r^r crossovers (see fig. 2).

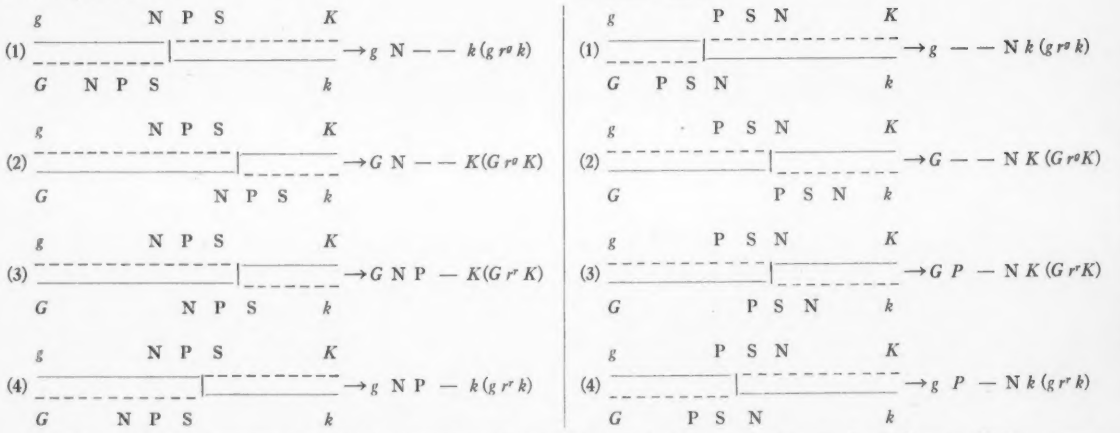
A With proximal N
B With distal N
(1) g N P S K → g N — — k (g r⁰ k)
G N P S k
(2) g N P S K → G N — — K (G r⁰ K)
G N P S k
(3) g N P S K → G N P — K (G r⁺ K)
G N P S k
(4) g N P S K → g N P — k (g r⁺ k)
G N P S k
(1) g P S N K → g — — N k (g r⁰ k)
G P S N k
(2) g P S N K → G — — N K (G r⁰ K)
G P S N k
(3) g P S N K → G P — N K (G r⁺ K)
G P S N k
(4) g P S N K → g P — N k (g r⁺ k)
G P S N k

FIGURE 2.—Unequal crossovers expected in the compound $g\ R^r\ K/G\ R^r\ k$ with either proximal or distal null elements. (Only crossovers yielding seed-color mutants are considered.)

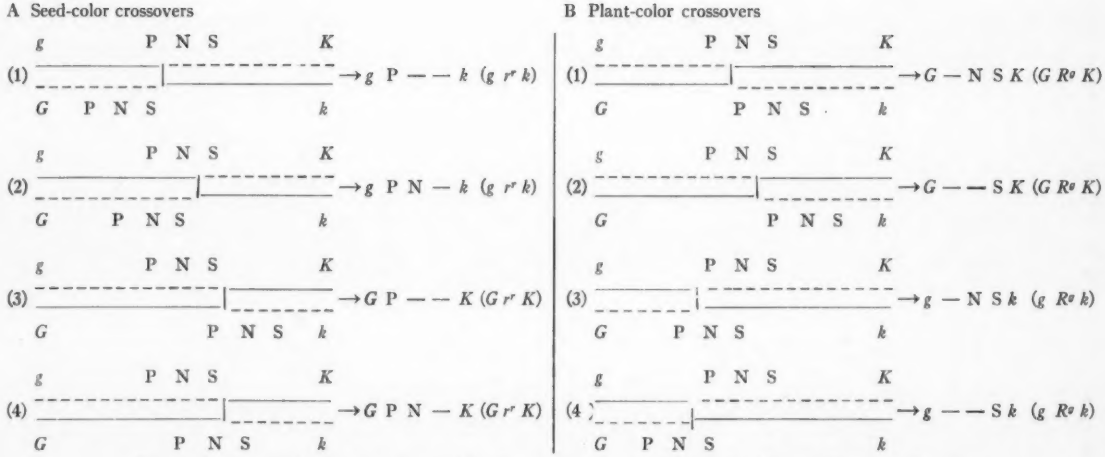
A Seed-color crossovers
B Plant-color crossovers
g P N S K
G P N S k
(1) → g P — — k (g r^r k)
(2) → g P N — k (g r^r k)
(3) → G P — — K (G r^r K)
(4) → G P N — K (G r^r K)
(1) → G — N S K (G R^g K)
(2) → G — — S K (G R^g K)
(3) → g — N S k (g R^g k)
(4) → g — — S k (g R^g k)

FIGURE 3.—Unequal crossovers expected in the compound *g* R^r *K*/*G* R^r *k* with a median null element.

The trials so far made with *g* R^r *K/G* R^r *k* have given no r^g crossovers (STADLER and NUFFER 1953). The five colorless seed crossovers produced by this compound were in fact of type *G* r^r *K*, but the sample was too small to give any significant indication of the existence of such elements.

There is no evidence at present which excludes the possibility of null elements between (P) and (S). In experiments conducted with the homozygote, median N elements would be undetectable since the unequal crossovers would be all of the r^r crossover type (see fig. 3A). However, the subsequent behavior of plant-color crossovers should give evidence of median N elements. The homozygous (PNS) compound is expected to produce two classes of unequal crossovers among the plant-color mutants, the (–NS) and the (– –S) types (see fig. 3B). In the case of the R^g crossover of (–NS) constitution, the (–NS)/(PS) compound should yield unequal crossovers among seed-color mutants showing both proximal and distal displacement. The (– –S)/(PS) compound, which represents the other type of R^g crossover, should yield seed-color crossovers showing proximal but not distal displacement.

Thus the critical criteria by which to detect the presence and the specific location of these null elements would be as follows: (PS), absence of r^g crossovers in homozygous (PS) plants; (NPS) or (PSN), presence of r^g crossovers in homozygous (NPS) or (PSN) plants; and (PNS), absence of r^g crossovers in the homozygote (PNS) and the presence of two types of R^g crossovers.

The results of the tests of the seed-color mutants produced by the compound *G* R^g-14 *k/g* R^r *K* are summarized in table 1. To calculate the most probable frequency of the noncrossover and crossover mutants the rate determined is stated in terms of the population in which analysis of the mutants was made. That is, 22 mutants were found, but only 13 of the 22 survived; thus the population of gametes tested is $13/22 \times 59{,}374 = 35{,}085$.

In a population of 35,085 gametes, this compound yielded 12 r^r mutants and one

TABLE 1

Frequency of noncrossovers and unequal crossovers among seed-color mutants in female gametes
G R^g-*14* k/g R^r *Cornell* K × g r^g k/g r^g k

Culture	Number of seeds examined	Number of colorless seeds	Fraction determined	Genotype of the mutant individuals					
				Noncrossovers			Unequal crossovers		
				g r^r *K*	*G* r^r *K*	*G* r^g	*k* r^r *k*	*G* r^r *k*	*G* r^g *K*
72:365	11,880	6	6/6	3	1*	0	2	0	0
366	8,052	4	4/4	2	0	0	1	1	0
73:1721	14,302†	2 + 1?	1/2 + 1?	0	0	0	1	0	0
1747	11,704†	5 + 1?	1/5 + 1?	0	0	1	0	0	0
1749	13,436†	3	1/3	0	0	0	1	0	0
Total	59,374	20 + 2?	13/20 + 2?	5	1	1	5	1‡	0

Adjusted mutation frequency 13/35,085.

* Intermediate mutant with faint aleurone color and a coincident crossover for *g*.

† Total population includes plants without *K* marker.

‡ Unequal crossover with a coincident crossover for *g*.

r^g mutant. Of the 12 r^r mutants found, five were r^r K noncrossovers, one was a noncrossover r^r K with faint aleurone color, and six were r^r k crossovers. The only seed-color mutant lacking plant color was a noncrossover G r^g k mutant.

The evidence that these mutants are unequal crossovers comes from the fact that only 1 percent of mutants analyzed should show a coincident crossover between R^r and the knob. The occurrence of six crossovers in this region among the 13 mutants investigated is highly significant evidence of a relation of crossing over to mutation ($P < 0.01$). The number of mutants expected by coincidence is 0.13.

The critical crossover class, the r^g K type, was absent in this culture, although with equal frequency of occurrence of the two crossover types, 75 percent of the crossovers detected should be of this type, since the female transmission ratio for chromosomes with and without the knob 10 was approximately 75 percent K:25 percent k. This knob ratio was determined by seedling tests. The number of crossovers identified, in comparison with numbers expected on the basis of a 75 percent K:25 percent k ratio, is as follows:

	Total number of crossovers	r^g K crossovers	r^r k crossovers
Observed	6	0	6
Expected		4.5	1.5

Stevens' statistical tables (1942) can be used to determine whether the observed distribution of 0 r^g K: 6 r^r k crossovers deviates significantly from the expected distribution of 4.5 r^g K: 1.5 r^r k crossovers. According to Stevens' table, for $n = 0$, $1/N = 1/6 = 0.166$, the upper limit of expectation of zero mutants at the $P = 0.05$ level is 2.83. Thus the difference is clearly significant. The absence of the crossover class produced by proximal displacement of the K-bearing segment can be accounted for by assuming the R^g-14 is deficient for the element (P), that is, that this mutant is of the (–S) type.

The proportion of unequal crossovers among the r^r mutants in the G R^g-14 k/g R^r K compound, uncorrected for the knob ratio, is six out of 12, or 50 percent (table 2A). The relative frequency of mutant types adjusted for the transmission advantage of the chromosome bearing K is given in table 2B. Thus the frequency in megaspores, before preferential segregation shows that the number of r^r k crossovers was 24, and the number of noncrossover mutants was eight r^r and four r^g. The corrected proportion of unequal crossovers among the r^r mutants is 24 out of 32, or 75 percent.

These results indicate that the excess of r^r over r^g mutants is due largely to unequal crossing over which increases the apparent rate of mutation of the opposite R^r Cornell allele.

The experimental results presented above are incompatible with hypothesis A which ascribes the reduced mutation rate of (S) wholly to a change in stability of the genic element (S). The data, however, do not differentiate between hypotheses B and C. Such a differentiation requires data from backcross of G R^g-14 k/G R^r k × g R^r K/g R^r K, which permits direct comparisons of mutation frequency in sib plants of G R^r k/g R^r K and G R^g-14 k/g R^r K constitution. At the present time there are no data available from backcross progeny marked for unequal crossover determinations, but there are data available from several cultures without crossover

TABLE 2

Proportion of unequal crossovers among r^r *mutants in* G R^g-*14* k/g R^r K

A. Uncorrected for knob ratio

Adjusted mutation frequency	13/35,085
r^r mutants obs.	12
r^g mutants obs.	1
r^r crossovers obs.	6
r^g crossovers obs.	0
r^r noncrossovers obs.	6
r^g noncrossovers obs.	1

Proportion of r^r crossovers among r^r mutants = 6/12 = 50%.

B. Corrected for knob ratio

	r^r K noncrossovers	r^r k crossovers	r^g k noncrossovers	n
Number observed in megaspores	6	6	1	35,085
Number occurring in megaspores to yield above with 75 K:25 k ratio	8	24	4	140,340

Proportion of r^r crossovers among r^r mutants = 24/32 = 75%.

markers. Inefficient as these tests are, they do permit a tentative choice between hypotheses B and C.

The backcross progeny tested for the relative frequency of r^r and r^g mutations, which are shown in tables 3 and 4, were produced by crossing two different R^g-14 k/R^r k F_1 plants (40:351 and 69:210) by homozygous R^r Cornell. These backcross cultures were grown in a detasseled plot with r^g g pollen. The genotype of each plant, as to R^g/R^r and R^r/R^r, was determined by seedling tests.

These results illustrate the two peculiarities of the R^g-14 allele. The data of tables 3 and 4 show the pronounced depression of the R^g mutation frequency and the M_G effect in the backcross progeny. In table 3 the total mutation frequency in R^g/R^r plants was 39/42,075, the mutations including 33 to r^r and 6 to r^g. The total muta-

TABLE 3

Seed-color mutation rate of R^g-*14*/R^r *Cornell versus parent* R^r *Cornell*/R^r *Cornell in female gametes*

F_1 Culture	Genotypic class		R^g/R^r fraction determined	Mutants from R^g/R^r plants	
	R^g/R^r	R^r/R^r		r^g	r^r
40:351–4	14/10,719	4/7,925	12/14	2	10
351–6	6/11,424	8/8,239	5/6	1	4
351–7	23/24,247	12/12,645	22/23	3	19
Total	43/46,390	24/28,809	39/43	6	33

Adjusted mutation frequency 39/42,075.

TABLE 4

Seed-color mutation rate of R^g-*14*/R^r *Cornell versus parent allele* R^r *Cornell*/R^r *Cornell in female gametes*

F_1 culture	Genotypic class		R^g/R^r fraction determined	Mutants from R^g/R^r plants	
	R^g/R^r	R^r/R^r		r^g	r^r
69:210–5	22/13,428	22/11,904	19/22	1	18
210–6	21/10,655	17/9,394	19/21	1	18
210–7	28/15,524	20/15,454	25/28	0	25
Total	71/39,607	59/36,752	63/71	2	61

Adjusted mutation frequency 63/35,144.

tion frequency, which is reported in table 4, was 63/35,144. Of the 63 mutants tested, 61 were r^r and only 2 were r^g. These data probably represent extremes of r^r:r^g ratio in the heterozygotes examined.

As mentioned previously, the M_G effect applies to the increased mutation rate of the R^r allele in the R^g/R^r class over the rate in the R^r/R^r class. Of the 42,075 gametes tested in the R^g/R^r plants (table 3), about one half of the gametes were tests of the R^r allele and one half were tests of the R^g allele. Thus the mutation rate for R^r in plants of R^g/R^r constitution was 15.68×10^{-4} (33/21,037.5); in plants of R^r/R^r constitution was 8.33×10^{-4} (24/28,809). The latter rate was calculated from the total population, since each seed tested an R^r gamete. In table 4, the observed mutation rate in plants of R^g/R^r constitution was 34.72×10^{-4} (61/17,572), while in plants of R^r/R^r constitution the rate was 16.05×10^{-4} (59/36,752). Thus the R^r mutation frequency was approximately twice as frequent in R^g/R^r plants as in R^r/R^r plants. These data may be interpreted in terms of hypothesis B.

Hypothesis B involves the assumption that the stability of (S) is not changed, but the effects of R^g-14 are due entirely to unequal crossing over. We may assign identical noncrossover mutation rates to R^r and R^g-14 and then calculate how large a proportion of the r^r mutants from R^g-14/R^r plants must be unequal crossovers. The frequency of mutation of the R^g-14 gene is taken as a measure of the gene mutation rate, assuming that all the r^g mutants observed are from mutations of the R^g allele and all are noncrossovers. If this speculation is wrong, the proportion r^r mutants due to unequal crossing over is still higher.

If the assumptions from hypothesis B were valid, the data of table 3 will show an expected number of noncrossover mutations for both R^r and R^g to be six, since the number of r^g mutants derived from the R^g allele is six (table 5A). Of the 33 mutants observed in heterozygous plants, the expected distribution of r^r crossovers and r^r noncrossovers will be 27 and six respectively. It is expected, therefore, that the 24 r^r mutants observed in the homozygous R^r plants will include 16 r^r crossovers and approximately eight r^r noncrossovers. Thus the percentage of r^r mutations ascribed to unequal crossing over will become 82 percent in the heterozygote and 67 percent in the homozygote.

The data of table 4 show two mutations of R^g-14 to r^g. Under hypothesis B, therefore, the 61 r^r mutants observed in the heterozygote will include 59 r^r crossovers

TABLE 5

Relative frequency or unequal crossovers among r^r mutants in R^g-14/R^r and R^r/R^r sibs
Hypothesis B

A. *Data from table 3*

	R^g/R^r	R^r/R^r
Mutation frequency	43/46,390	24/28,809
Adjusted frequency	39/42,075	
r^r mutants obs.	33	
r^g mutants obs.	6	
r^r crossovers exp.	27	
r^r noncrossovers exp.	6	8^+
r^g noncrossovers exp.	6	

Proportion of crossovers among r^r mutants in R^g/R^r plants = 27/33 = 82%.
Proportion of crossovers among r^r mutants in R^r/R^r plants = 16/24 = 67%.

B. *Data from table 4*

	R^g/R^r	R^r/R^r
Mutation frequency	71/39,607	59/36,752
Adjusted frequency	63/35,144	
r^r mutants obs.	61	
r^g mutants obs.	2	
r^r crossovers exp.	59	
r^r noncrossovers exp.	2	4^+
r^g noncrossovers exp.	2	

Proportion of crossovers among r^r mutants in R^g/R^r plants = 59/61 = 97%.
Proportion of crossovers among r^r mutants in R^r/R^r plants = 55/59 = 94%.

and two r^r noncrossovers; in the homozygote the expected number of r^r crossovers and noncrossovers is 55 and 4 respectively. Thus 97 percent of the r^r mutants in the heterozygote and 94 percent of the r^r mutants in the homozygote may be attributed to the occurrence of unequal crossing over.

The same data may be interpreted in terms of hypothesis C. This hypothesis involves the assumption that the stability of (S) is affected by mutation or loss of (P), and the occurrence of unequal crossing over increases the number of r^r mutants in the heterozygote. The percentage of r^r mutants that must be ascribed to unequal crossing over in the R^g/R^r class may be derived from the mutation rate of r^r mutants in the R^r/R^r plants. In this case, the gene mutation frequency of R^r will be identical in the heterozygote and the homozygote. This implies that the r^r mutants from the R^r/R^r are all noncrossovers and that there is a similar occurrence of r^r noncrossovers from R^g-14/R^r.

But the assumption of no crossing over in R^r/R^r plants is not entirely plausible, since results already at hand indicate that crossover mutants occur in the homozygote (STADLER and NUFFER 1953). These were at a rate of 7×10^{-5} and constituted about one third of the r^r mutants. Their relative frequency among r^r mutants, however, must be calculated on a different basis for comparison with those

TABLE 6

Relative frequency of unequal crossovers among r^r *mutants in* R^g–*14*/R^r *and* R^r/R^r *sibs*
Hypothesis C

A. *Data from table 3*

Adjusted mutation frequency for r^r mutants in R^g/R^r	33/42,075
Mutation frequency in R^r/R^r	$24/28{,}809 = 8.33 \times 10^{-4}$
r^r noncrossovers exp. in R^g/R^r	17.5
$\frac{8.33}{10{,}000} = \frac{X}{21{,}037.5}$	
r^r crossovers exp. in R^g/R^r	15.5

Proportion of crossovers among r^r mutants in R^g/R^r plants = 15.5/33 = 47%.

B. *Data from table 4*

Adjusted mutation frequency for r^r mutants in R^g/R^r	61/35,144
Mutation frequency in R^r/R^r	$59/36{,}752 = 16.05 \times 10^{-4}$
r^r noncrossovers exp. in R^g/R^r	28.2
$\frac{16.05}{10{,}000} = \frac{X}{17{,}572}$	
r^r crossovers exp. in R^g/R^r	32.8

Proportion of crossovers among r^r mutants in R^g/R^r plants = 32.8/61 = 54%.

in the heterozygote, since in the $G\ R^r\ k/g\ R^r\ K$ compound there are two chromosomes with R^r instead of only one. The fact that crossovers and noncrossovers are distinguishable here only by g classification badly weakens the data, since one seventh of the crossovers may be misclassified due to coincident crossing over between G and R. Thus the percentage of crossovers among r^r mutants, very inaccurately estimated, is 38 percent. This is low, but the determination has practically no significance.

The data of table 3 show that the mutation rate of R^r Cornell in R^r/R^r plants is 8.33×10^{-4} (24/28,809). If the assumptions from hypothesis C were correct, the expected number of r^r noncrossovers in plants of R^g/R^r constitution will be 17.5 (table 6A). This hypothesis implies that there is an equal mutation rate of r^r noncrossovers in R^r/R^r and R^g/R^r. Of the 33 r^r mutants observed in the heterozygote, 17.5 will be noncrossovers and here, 15.5 will be crossovers. Thus the proportion of r^r crossovers to the total number of r^r mutants in the heterozygote becomes 15.5/33 (or 47 percent). The reduction in gene mutation rate of the element (S) is from 8.33×10^{-4} prior to mutation of (P) to 2.84×10^{-4} following mutation of (P).

Applying the assumptions from hypothesis C to the data in table 4, the number of noncrossover mutants of R^r in R^g/R^r plants becomes 28.2, since the mutation rate of R^r in R^r/R^r plants is 16.05×10^{-4}. Thus the observed number of 61 r^r mutants in R^g/R^r plants will include 32.8 r^r crossovers and 28.2 r^r noncrossovers. The proportion of r^r mutations ascribed to unequal crossing over becomes 32.8/61 (or 54 percent). In this case, the reduction in gene mutation rate of the element (S) is from 16.05×10^{-4} to 1.13×10^{-4}. Thus the gene mutation rate of R^r may be much

higher than that of R^g-14, even though the apparent increased rate of R^r mutations in R^g-14/R^r is due to crossing over.

DISCUSSION

Three hypotheses have been proposed to account for the lowered mutation rate of R^g-14 in the heterozygote R^g-14/R^r and the pronounced increase in r^r mutation frequency of R^g-14/R^r over R^r/R^r.

The data reported on the compound *G* R^g-14 *k/g* R^r *K* (table 1) give little support to hypothesis A, which ascribes the effects of R^g-14 to a change in mutability of (S). Of the 12 r^r seed-color mutants found, six were r^r crossovers. This experiment indicates that the increased frequency of r^r mutations in the heterozygote is due largely to the occurrence of unequal crossing over.

The results, however, do not differentiate between hypotheses B and C. In order to distinguish between these two hypotheses, data are required on the relative mutation frequency of r^r mutations in knob-marked comparisons of R^g-14/R^r and R^r/R^r sibs. These results are not yet available, but some indication of the percentage of r^r mutations due to unequal crossing over may be obtained from backcross progeny unmarked for the determination of unequal crossing over.

Under the hypothesis B, which assumes no change in the stability of (S) following loss of (P), but rather implies that the effects are due entirely to unequal crossing over, the gene mutation frequency of R^r would be as low as that found in R^g-14. This is illustrated in table 5 with data from tables 3 and 4. In table 3 the expected percentage of crossovers among r^r mutations would be 82 percent in the heterozygote and 67 percent in the homozygote. The proportion of unequal crossovers becomes still higher when data from table 4 are considered. In this case the percentage of unequal crossovers would include 97 percent of the r^r mutations in the R^g-14/R^r plants and 94 percent in the homozygous R^r sibs. Therefore, this hypothesis would seem to be questionable for the reason that it requires an unreasonably high frequency of unequal crossing over.

Hypothesis C assumes that the stability of (S) is affected both by mutation or loss of (P) and by unequal crossing over. This hypothesis implies that the frequency of unequal crossing over among mutants in the R^g/R^r class will be lower, to an extent corresponding to the increased frequency of noncrossover mutation of R^r/R^r. Its minimal value may be calculated by using the mutation rate of R^r/R^r determined in accounting for the r^r noncrossover mutations from the R^g-14/R^r class. In table 4 with a high rate of R^r/R^r mutation (16.05×10^{-4}), the proportion of r^r mutants due to unequal crossing over need not be high (i.e. 32.8/61 or 54 percent), while the assumption of full effect would require an extremely high percentage (97 percent). Thus it appears more likely that many of the r^r mutants in the R^g-14/R^r plants represent gene mutations rather than unequal crossovers. At the present time it cannot be concluded that hypothesis C is more valid than hypothesis B, since hypothesis C is based on the assumption that unequal crossing over is absent in the homozygote R^r/R^r. Data already at hand indicate that some unequal crossing over occurs in the homozygote.

A more definite basis for estimating the reduction in gene mutation rate for the

(S) element may be obtained from a direct comparison of noncrossover r^r mutation frequency in *G* R^r K/g R^r k and r^g noncrossover mutation frequency in *G* R^g-14 K/g R^g-14 k sibs. The M_G effect may also be measured directly in *G* R^g-14 k/g R^r K and *G* R^r k/g R^r K sib comparisons. Both of these tests are now in progress.

If hypothesis C were valid, the question might be asked, why should the compound (–S)/(PS) give more frequent crossovers than (PS)/(PS)? The high frequency of unequal crossing over could be due to the mode of pairing in (–S)/(PS) compound. Pairing initiated proximally would, in the R^g/R^r compound, result in $\overline{\text{S}}$PS with opportunity for crossing over, while pairing initiated distally would result in PS without this opportunity. Both types, in the R^r/R^r compound, would result in $\overline{\text{PS}}$. In the latter compound, therefore, unequal pairing would be expected only when pairing is initiated at the (S) or (P) locus.

An additional feature of considerable interest in the analysis of R^g-14 is the indication that the gene R^g-14 is deficient for the element (P). This type would be expected to result from a crossover mutation in the parent R^r Cornell allele. It does not necessarily follow that noncrossover mutations would be all (pS), since the loss of (P) could be due to some mechanism which is independent of crossing over. If, however, R^g crossovers differ from R^g noncrossovers by the physical loss rather than by recessive mutation of element (P), it may be possible to demonstrate by the unequal crossover mutants resulting from pairing differences the presence of an element (p) in the R^g noncrossovers and its absence in R^g crossover alleles. This would also provide a criterion for distinguishing between gene mutation and physical loss in other R^g mutations, specifically those induced by irradiation. Several R^g mutants of known crossover origin are being tested at the present time.

SUMMARY

The occurrence of unequal crossing over in the *G* R^g-14 k/g R^r K compound indicates that the R^g-14 gene is deficient for the element (P). Presumably the plant-color element was lost by the occurrence of unequal crossing over in the parent allele.

Three alternative hypotheses were proposed to account for the peculiarities of R^g-14 behavior which indicated both the lowered mutation rate of the element (S) and the M_G effect. Hypothesis A implied that the peculiarities of R^g-14 were due to a change in mutability of the element (S) while in B they were due to unequal crossing over and in C to unequal crossing over as well as a change in mutability of (S).

Hypothesis A was excluded since 50 percent of the r^r mutants from the *G* R^g-14 k/g R^r K compound were unequal crossovers. Application of hypothesis B to backcross data showed an unreasonably high frequency of unequal crossovers in both the R^g/R^r and R^r/R^r plants while hypothesis C demonstrated a moderate frequency of unequal crossovers in R^g/R^r and R^r/R^r sibs. These data, however, do

not permit a definite choice between hypotheses B and C since the stocks were unmarked for the detection of unequal crossovers.

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EFFECTS OF LINKAGE ON THE COVARIANCES BETWEEN RELATIVES[1]

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WITH two alleles per locus, partitioning of the hereditary variance of diploid populations factorially into additive, dominance, additive by additive and higher order components depends only on the assumption that genotypic frequencies are proportional to the marginal frequencies of the phases (AA, Aa or aa) of each locus (COCKERHAM 1954). This assumption, in effect, specifies linkage equilibrium. When an arbitrary number of alleles at each locus is considered, the variance can be broken into the same framework of partitions at least when an additional assumption of Hardy-Weinberg equilibrium of marginal frequencies is met (KEMPTHORNE 1954). Thus, in randomly mating populations linkages do not affect the hereditary variance nor its partitions as long as the population is in linkage equilibrium. Even so, linkages do affect the covariances between some relatives. Covariances between relatives are the sources from which components of hereditary variance are estimated. While the breeder or geneticist may be willing to assume linkage equilibrium in his population, he is certainly not willing to assume that none of the loci are linked. The purpose of this paper is to elucidate some of these linkage effects on the covariances and to indicate relatives whose covariances will be affected. Linkage is used in the sense of lack of free recombination at meiosis. Position effects are assumed to be absent. Only relatives in randomly mating populations that are in linkage equilibrium will be considered.

The method of obtaining the covariance between relatives makes use of MALECOT's (1948) probabilities of genes being identical by descent which were employed by KEMPTHORNE (1954). The basic result, as given by KEMPTHORNE, is that the coefficients of the various types of components of variance in the covariances between relatives are a function of, and can be computed from, the probabilities of genes having descended from the same gene. For example, consider two half sibs which have descended from a sire with the following genotype,

$$\frac{x_1 \; \rho_{12} \; y_1 \; \rho_{23} \; z_1}{x_2 \qquad y_2 \qquad z_2},$$

where x_1 and x_2, y_1 and y_2, and z_1 and z_2 are alleles at the x, y and z locus, respectively, and ρ_{12} and ρ_{23} are recombination frequencies. Now in the covariance between half sibs, the coefficient of the additive component of variance arising from any locus, the x locus in this example, is

$$\frac{\phi}{2} = \frac{P(s, s' = x_1) + P(s, s' = x_2)}{2} = \frac{1}{4}.$$

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$P(s,s' = x_1) = \frac{1}{4}$ is the probability that both half sibs, s and s', inherited the allele, x_1, and so on. Thus ϕ is the total probability that both half sibs inherited the same gene from their sire. No components involving dominance enter into the covariance between half sibs because dominance components enter into covariances between relatives only when they have common dominance elements; that is, the two genes at a locus of one relative are identical by descent with the two genes at the same locus of the other relative.

With free recombination, $\rho_{12} = \rho_{23} =$ etc. $= \frac{1}{2}$, the coefficient of the two-factor additive component arising from any pair of loci in the covariance between half sibs is $(\phi/2)^2$. However, to include linkage the appropriate coefficient is

$$\frac{\phi_{12}}{4} = \frac{P(s, s' = x_1 y_1) + P(s, s' = x_2 y_2) + P(s, s' = x_1 y_2) + P(s, s' = x_2 y_1)}{4}$$

These probabilities are, in their respective order,

$$\phi_{12} = \left(\frac{1 - \rho_{12}}{2}\right)^2 + \left(\frac{1 - \rho_{12}}{2}\right)^2 + \left(\frac{\rho_{12}}{2}\right)^2 + \left(\frac{\rho_{12}}{2}\right)^2$$

$$= \frac{1}{4} + \frac{(1 - 2\rho_{12})^2}{4} = \frac{1 + \delta_{12}}{4}$$

Thus $\phi_{12} = \phi^2$ only if there is no linkage. The coefficient of the two-factor additive component, $\sigma^2_{a_1a_2}$ for the x and y loci in this case, in the covariance between half sibs is $(1 + \delta_{12})/16$. Linkage causes a positive bias in the amount of $\delta_{12}\sigma^2_{a_1a_2}/16$. This bias is summed over all pairs of linked loci, $\Sigma\delta_{ij}\sigma^2_{a_ia_j}$.

For the triple additive components the coefficients in the covariance between half sibs are $(1 + \delta_{ij})(1 + \delta_{jk})/64$, where the loci are in the linear order of i, j and k, and provided interference is absent, i.e., $\rho_{ik} = \rho_{ij} + \rho_{jk} - 2\rho_{ij}\rho_{jk}$ or $\delta_{ik} = \delta_{ij}\delta_{jk}$. Less than free recombination of only two of the loci still causes a positive bias. This observation can be generalized further to say that less than free recombination of two loci will bias the coefficients of all the additive epistatic components in which they are involved. For example, the coefficients for quadruple additive components are $(1 + \delta_{ij})(1 + \delta_{jk})(1 + \delta_{kl})/256$ and so on for the higher order components.

In the covariance between full sibs the coefficients of all types of epistatic components are affected by lack of free recombination. The coefficients of the various components are set out in table 1 for components up to 3 factors. The loci are considered to be in the linear order i, j and k, and interference is assumed to be absent. No simple method by which one could ascertain any desired coefficient was found. Again, the coefficient of any epistatic component is affected by linkage of any of the loci involved in the component, and the effect is always to increase the coefficient.

If position effects are absent, as assumed throughout, the covariance between parent and offspring is unaffected by linkage. This can be seen for two loci by noting that the coupling and repulsion parents have identical genotypic values and that the distribution (and consequently the mean) of their offspring as a group does not depend on linkage so that the average product or covariance between parent and

TABLE 1

Coefficients of components of hereditary variance in the covariance between full sibs

Type of component	Coefficients		
	Unspecified recombination	No recombination	Free recombination
a_i	1/2	1/2	1/2
d_i	1/4	1/4	1/4
$a_i \times a_j$	$[1 + (1 + \delta_{ij})]/8$	3/8	1/4
$a_i \times d_j$	$(1 + \delta_{ij})/8$	1/4	1/8
$d_i \times d_j$	$(1 + \delta_{ij})^2/16$	1/4	1/16
$a_i \times a_j \times a_k$	$[1 + (1 + \delta_{ij})\ (1 + \delta_{jk})]/16$	5/16	1/8
$a_i \times a_j \times d_k$	$[(1 + \delta_{ij})(1 + \delta_{jk}) + (1 + \delta_{ik})(1 + \delta_{jk})]/32$	1/4	1/16
$a_i \times d_j \times a_k$	$(1 + \delta_{ij})(1 + \delta_{jk})/16$	1/4	1/16
$d_i \times a_j \times a_k$	$[(1 + \delta_{ij})(1 + \delta_{jk}) + (1 + \delta_{ij})(1 + \delta_{ik})]/32$	1/4	1/16
$a_i \times d_j \times d_k$	$(1 + \delta_{ij})(1 + \delta_{jk})^2/32$	1/4	1/32
$d_i \times a_j \times d_k$	$(1 + \delta_{ij})(1 + \delta_{ik})(1 + \delta_{jk})/32$	1/4	1/32
$d_i \times d_j \times a_k$	$(1 + \delta_{ij})^2(1 + \delta_{jk})/32$	1/4	1/32
$d_i \times d_j \times d_k$	$(1 + \delta_{ij})^2(1 + \delta_{jk})^2/64$	1/4	1/64

offspring remains the same. The lack of repulsion types from the coupling parent is supplied by an excess of repulsion types from the repulsion parent, and so on. This same condition holds for all other sets of relatives where one relative is an ancestor of the other. Thus in randomly mating diploid populations where genotypic frequencies are in linkage equilibrium, the covariances between parent and offspring, grandparent and grand offspring, great grandparent and great grand offspring and so on are unaffected by linkage.

Sets of relatives whose covariances are affected by linkage are those in which one is not a common ancestor of the other, e.g., half sibs, full sibs, cousins, uncle and nephew. As the number of generations from one relative through the common ancestor to the other relative increases the amount of bias due to linkage decreases. At the same time, however, the ratio of the amount of bias to the variance found without linkage increases. For example, these ratios are δ_{12}, $3\delta_{12}$ and $6\delta_{12} + \delta_{12}^2$ for the two factor additive components in the covariances of half sibs, uncle and nephew and half first cousins, respectively.

The contribution of linkage to the covariances between relatives can not be generally foretold of course. Of the covariances which are subject to linkage bias only the epistatic components are affected and these only if they involve loci which have a recombination frequency less than one half. Agencies which limit recombination, such as localization of chiasmata, crossover suppressors and inversions could cause considerable bias in the covariances.

Methods of estimating various components of hereditary variance must necessarily involve covariances between relatives. If linkage effects are not accounted for directly in the analysis or avoided by choosing relatives whose covariances are unaffected, then they must at least be regarded as a probable source of bias in the results.

SUMMARY

In randomly mating diploid populations whose genotypic frequencies are in linkage equilibrium, the covariances between relatives of which one is an ancestor of the other are not affected by linkages, provided position effects are absent. In contrast, covariances between relatives where one is not an ancestor of the other are affected by recombination frequencies less than one half. The lower the recombination frequency, the higher the covariance, and the bias is in the amount of the epistatic components that appears in the covariances and not in the amount of additive genetic and dominance components. Explicit expressions are given for full sibs and half sibs up to and including three factor epistatic components.

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TRANSDUCTION IN ESCHERICHIA COLI K-12[1]

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A SYSTEM of genetic transduction has been discovered in the sexually fertile K-12 strain of *Escherichia coli*. This transduction is mediated by lambda, a temperate phage for which K-12 is normally lysogenic.

The distinctive features of the lambda-K-12 system include the following: (1) The transductions are limited to a cluster of genes for galactose fermentation. The *Gal* loci are closely linked to each other and to *Lp*, the locus for lambda-maintenance. (2) The transducing competence of lambda depends on how it is prepared. Competent lambda is produced by induction of lysogenic bacteria; lambda harvested from infected, sensitive hosts is incompetent. (3) The transduction clones are often heterogenotic, that is, heterozygous for the *Gal* genes which they continue to segregate. Technical advantages of the lambda system include recombinational analysis by the sexual cycle and the availability of lysates in which nearly every lambda particle is competent.

MATERIALS AND METHODS

Cultures

The origin and history of the *Escherichia coli* K-12 cultures studied have already been described (E. LEDERBERG 1950, 1952; LEDERBERG and LEDERBERG 1953). The emphasis will be placed here on the *Gal* loci (+ = fermenting galactose; − = nonfermenting) and on the locus which controls the maintenance of lambda (Lp_1).

The phenotypes of cultures with different alleles of Lp_1 are as follows:

	Lysed by lambda	Lyses Lp_1^s culture
Lp_1^s culture (sensitive)	yes	no
Lp_1^+ culture (lysogenic)	no	yes
Lp_1^r culture (immune)	no	no

Regardless of their Lp_1 genotype, cultures have been found to adsorb lambda. Thus Lp_1^+ and Lp_1^r are resistant to lysis by lambda in spite of their ability to adsorb the phage. In contrast with this, mutants resistant to lambda-2, a virulent mutant of lambda, are resistant because they do not adsorb either lambda or lambda-2 under the experimental conditions used here.

Media

The media used include: broth, Difco penassay; agar for phage assay, Difco nutrient agar with 0.5 percent NaCl; indicator medium, EMB agar plus one percent

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sugar; minimal agar, D(O); and minimal indicator agar, EMS (J. LEDERBERG 1950). Special supplements were added where indicated. All dilutions of phage lysates were made in either penassay or nutrient saline broth, and cell suspensions were diluted in either 0.5 percent saline or penassay broth.

General methods

Plates and tubes were incubated at 37°C. When high cell densities were desired, broth cultures were aerated by bubbling filtered air through them. Propylene glycol monolaurate (Glyco Products Co., Inc.) at a final concentration of 0.01 percent was added to bubbled cultures to lessen foaming. Phage assays were made either in agar layer or by spreading a portion of dilute lysate with *Gal*$^-$ cells on EMB galactose agar.

Lysates containing lambda in high titer were prepared by two methods: (1) "Induced lambda" was liberated from lysogenic bacteria after treatment with ultraviolet (UV) (WEIGLE and DELBRÜCK 1951); (2) "Lytic lambda" was harvested from sensitive bacteria infected with free lambda. The induced lambda was prepared as follows: aerated, penassay grown cultures of an *Lp*$^+$ strain (ca. 10^9 cells per ml) were sedimented in the centrifuge, the broth discarded, and the cells resuspended in 0.5 percent saline. The cell suspensions (10 ml) were exposed to the radiation from a GE Sterilamp (45 seconds at 50 cm) in open petri dishes on a platform shaker. After irradiation the suspensions were diluted with an equivalent volume of double strength penassay broth and aerated at 37°C until maximal clearing was obtained. This usually required from 2 to 3 hours. To produce lytic lambda, an inoculum of induced lambda was adsorbed on to penassay grown sensitive cells. After the adsorption period the cells were sedimented to separate them from the penassay broth and resuspended in nutrient saline broth. The suspension was then aerated until maximal clearing was obtained (4–5 hours). Induced lysates have phage plaque titers of about 3×10^{10} particles per ml, while lytic lysates have about 10^{10}.

Induced lambda was used in all experiments unless otherwise stated.

Methods for testing for transduction

In order to detect infrequent genetic changes, selective agar media were used: EMB agar for fermentation markers; EMB agar plus 100 micrograms per ml streptomycin for streptomycin resistance; minimal agar for nutritional markers. About 10^8 mutant cells in 0.1 ml broth or saline, and 0.1–0.2 ml of lysate were added to the surface of each agar plate and then spread with a bent glass rod. The plates were incubated 2–3 days before being scored.

Transduction clones selected by these methods develop in a heavy background of unchanged cells. On EMB medium, negative cells grow at the expense of the peptone; by using sugar as well, positive clones form papillate outgrowths from the negative background. EMB agar serves as an indicator as well as a selective medium; isolated positive colonies are deeply colored, while negative colonies remain translucent (illustrated in fig. 3).

The transduction clones were purified by the following procedure. Papillae were picked with a needle and suspended in 1 ml of sterile water. A loopful (ca. 0.001

ml) of this suspension was then streaked upon a portion of another plate of the EMB agar. These primary dispersals of the transduction clones were nearly always mixed. Direct picking and streaking, or spotting without any purification cannot be trusted. From the primary streaks a single colony that looked pure was picked to water and streaked as before. This operation was repeated once again, and a single colony from the last streaking was taken to represent the transduction clone. In addition to freeing the transduction clone from unchanged background cells, this method of purification may also act selectively within an unstable clone. Picking apparently pure colonies leads to an overestimate of the fraction of non-segregating clones.

RESULTS

The transductions

Although a number of different loci affecting diverse portions of the genotype were tested, only genes of a cluster of loci for galactose fermentation were transduced by lambda lysates (MORSE 1954). The *Gal* loci, of which about seven have been investigated thus far, are closely linked to one another (less than one percent recombination) and to Lp_1, the locus for lambda maintenance (LEDERBERG and LEDERBERG 1953, and unpublished).

The transformation of Gal^- cells to Gal^+ by induced lambda is illustrated in figure 1. Each papilla is a clone of galactose fermenting cells; on the area of the plate to which lysate was added, most of the Gal^+ papillae are transduction clones. The

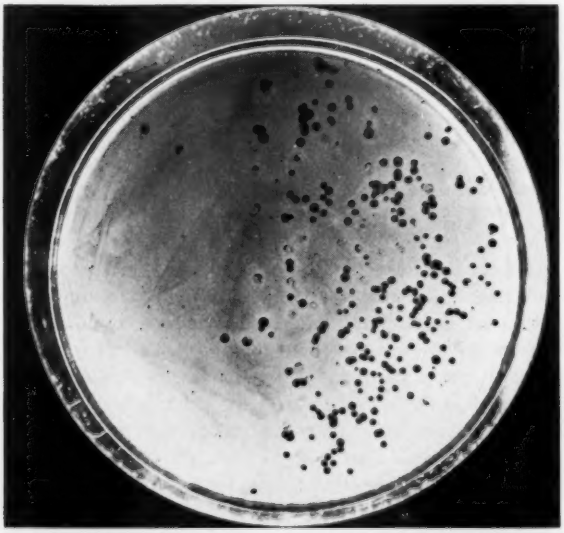

FIGURE 1.—The production of Gal^+ papillae from a Gal^- background of cells by a lambda lysate. Left, the control, no lysate added. Right, 0.1 ml of lysate from a Gal^+ culture. Some of the papillae have been picked with a needle.

TABLE 1

Transformation of Gal⁻ *cultures by lysates of* Gal⁺

Recipient culture	Lysate of:	Lambda titer in 10^{10} per ml	Number of *Gal*⁺ papillae		*Gal*⁺ papillae per 10^7 lambda
			Control (no lysate)*	0.1 ml lysate	
$Gal_1^-Lp^+$	Gal^+	1.4	2	405	2.9
	Gal_1^-	2.4	2	2	—
$Gal_2^-Lp^+$	Gal^+	1.4	20	356	2.4
	Gal_2^-	4.9	20	10	—
$Gal_4^-Lp^+$	Gal^+	1.4	47	394	2.5
	Gal_4^-	1.7	47	50	—
$Gal_4^-Lp^s$	Gal^+	1.4	4	2112	15.1
	Gal_4^-	1.7	163	86	—
$Gal_4^-Lp^s$†	Gal^+	2.3	10	3020	13.1
	Gal_4^-	1.7	10	18	—
$Gal_4^-Lp^s$†	Gal^+	2.3	5	1296	5.6
$Gal_4^-Lp^r$	Gal^+	2.3	40	161	0.5
$Gal_4^-Lp^r$‡	Gal^+	1.4	29	129	0.7
$Gal_4^-Lp^r$‡	Gal^+	1.6	28	92	0.4

* The *Gal*⁺ papillae on the control are spontaneous reversals of phenotype.

† Different stocks.

‡ Different experiments.

quantitative relationships are illustrated in figure 2. The data can be summarized: (1) Regardless of the Lp_1 genotype of the recipient, transductions were obtained; (2) with each genotype the number of transductions was proportional to the amount of lysate plated; (3) Lp^s recipient cultures gave 5 to 10-fold more papillae per unit of lysate than either Lp_1^+ or Lp_1^r. Further, the transducing activity of lysates (which contain 10^{10} lambda per ml) varies according to the number of cells plated: (1) with Lp_1^+ *Gal*⁻ cultures there is a two-fold increase between 10^6–10^9 cells per plate, with a plateau of maximal yield around 10^8 cells per plate; (2) Lp^s *Gal*⁻ recipient cultures show about a six-fold increase over a similar range of cell platings, with the highest yield at the highest cell density.

The transducing activity of lysates is specific; that is, a lysate of a Gal_x^- culture will not transform Gal_x^- cultures (table 1) but *Gal*⁺ papillae were found with a Gal_y^- culture. The specificity is extended further in that some galactose positive phenotypic reversals of a *Gal*⁻ culture give lysates with transducing activity on the original Gal_x^- indicator (table 2). The different types of phenotypic reversals may be understood under the following hypothesis: (1) reverse mutations (Gal_x^- to Gal_x^+) yield cultures that give active lysates, and (2) suppressor mutations (Gal_x^- Suppressor⁻ to Gal_x^- Suppressor⁺) will yield incompetent cultures when the suppressor lies outside the region transduced.

From the data in table 1 and figure 2 the ratio of the transducing particles to the lambda particles in a lysate may be obtained. Lp^s recipient cultures give about one transduction per 10^6 lambda; Lp^+ recipients, one per 10^7. One per 10^6–10^7 lambda will be referred to as LFT (low frequency of transduction).

TABLE 2

The action of lysates of reverse mutants

Lp$^+$ recipient culture	Lysate of reversion	Numbers of *Gal*$^+$ papillae observed	
		Control (no lysate)	0.1 ml lysate
Gal$_1^-$	*Gal*$_1^+$ #1	0	648
Gal$_2^-$	*Gal*$_2^+$ #1	10	96
	Gal$_2^+$ #2	6	552
Gal$_4^-$	*Gal*$_4^+$ #5	39	204
	Gal$_4^+$ #8	25	291

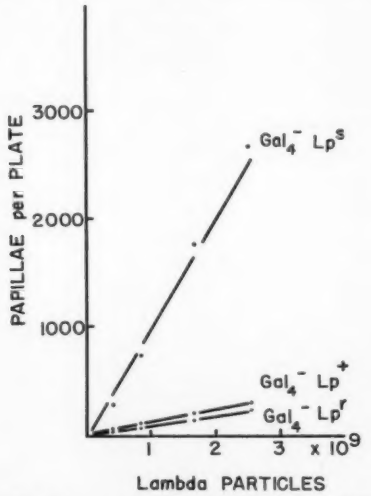

FIGURE 2.—Proportionality between amount of lambda lysate (LFT) plated and number of papillae formed from *Lp*^{s}, *Lp*$^+$ and *Lp*^{r} *Gal*$^-$ cultures. The ratio of papillae to lambda particles is 10^{-6} for an *Lp*^{s} culture, 10^{-7} for *Lp*$^+$ and *Lp*^{r} cultures.

Examination of the Gal$^+$ *clones formed by transduction*

After purification the transduction clones were examined for changes at loci other than the *Gal* series. A number of markers were examined, including fermentative, nutritional, and phage and drug resistance mutations. The only changes at other loci were *Lp*^{s} to *Lp*$^+$ in lambda sensitive recipients, and occasionally *Lp*^{r} to *Lp*$^+$ in lambda immune cultures. Transduction clones from *Lp*$^+$ recipients were invariably *Lp*$^+$.

To determine whether lysogeny was causally related to transduction, a reconstruction experiment was done. To a mixture of lysate and *Gal*$^-$ *Lp*^{s} cells, *Gal*$^+$ *Lp*^{s} cells labelled with a mutant character were added to estimate the frequency of chance lysogenization in the untransformed cells in a transduction mixture. After papillae had formed, they were picked, purified, and on the basis of the differential label, divided into: (1) the inserted *Gal*$^+$, and (2) the transductions. The frequency

TABLE 3

Comparison of the lysogenization of transformed and non-transformed sensitive strains: reconstruction experiment

Types recovered from mixture* of Lp^s bacteria and LFT lysate	Number of clones examined	Percent of clones lysogenized
Inserted $Gal^+Lac^+S^s$	46	68.5
Recipient $Gal^-Lac^-S^r$ (non-transformed)	40	72.5
Transduction $Gal^+Lac^-S^r$	103	100.

* 10^8 $Gal^-Lac^-S^r$, 100 $Gal^+Lac^+S^s$, 10^9 lambda particles.

of lysogeny was determined in the two classes, and in the Gal^- background. Whereas unchanged Gal^- cells and the inserted Gal^+ were each only 70 percent lysogenized, the transduction clones were 100 percent lysogenized (table 3).

When Lp^r cultures were used as recipients, 14/112 (12 percent) of transduction clones formed were Lp^+. Although the fraction is small, all previous attempts to lysogenize these cultures have been unsuccessful. The isolation of transduction clones evidently selects for these cells that have been infected with lambda particles from the input lysate.

The original Gal^+ strain and spontaneous reversions of the Gal^- mutants have all been stable in ordinary culture. However, the Gal^+ clones formed by transduction are unstable for galactose fermentation as shown by the recurrence of negative and mosaic colonies (fig. 3). Despite many serial single colony isolations the galactose-positives continue to segregate galactose negative progeny. They behave as if

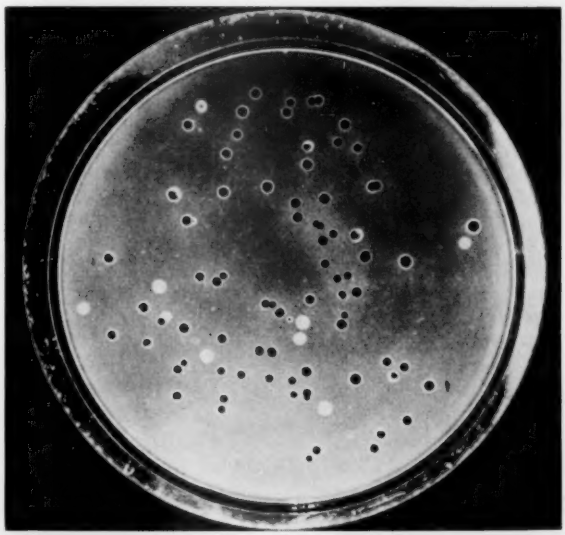

FIGURE 3.—EMB galactose agar plate spread with cells form a culture of a heterogenote, showing Gal^+, Gal^- and sectoring colonies.

TABLE 4

Frequency of instability for galactose fermentation among the transduction clones

Recipient cells	Unstable clones/total examined	Percent unstable
Gal_1^- Lp^s	9/22	41
Gal_1^- Lp^+	40/48	83
Gal_2^- Lp^+	22/24	92
Gal_4^- Lp^s	13/24	54
Lp^+	20/24	83
Lp^r	29/48	60
Gal_6^- Lp^s	6/8	75
Gal_8^- Lp^s	28/48	58
Lp^+	16/24	67

heterozygous for a single gene (or short chromosome segment) and may be designated as heterogenotes. Instability among the transduction clones is quite frequent; 484 of 609 clones (70 percent) were found unstable (representative data are given in table 4). This estimate is probably low because the purification procedure acts selectively against unstable clones.

The frequency of segregation has been estimated from the incidence of *Gal*$^-$ in small clones of heterogenotes. The probability of segregation per bacterial division is about 2×10^{-3} (table 5). By repeated reisolation, however, heterogenotic lines can be maintained indefinitely.

The segregants from the heterogenotic clones were examined with regard to their *Gal* and *Lp* character. Lysates of the segregants have no transducing activity on the *Gal*$^-$ culture that was used as the recipient in forming the transduction clone and are therefore allelic to it. The same lysates continue to give one transduction per 10^6–10^7 lambda (LFT ratio) on non-allelic *Gal*$^-$ cultures. With different recipient cultures the *Lp* alleles of the segregants were (1) *Lp*$^+$ recipient, all segregants *Lp*$^+$; (2) *Lp*^{s} recipients, all segregants *Lp*$^+$; (3) *Lp*^{r} cells, the segregants were usually *Lp*^{r}. In one instance, a heterogenote segregated both *Lp*$^+$/*Lp*^{r} and *Gal*$^+$/*Gal*$^-$.

Lysates prepared from the heterogenotes have two outstanding features: (1) instead of containing 10^{10} lambda particles per ml, they seldom have titers higher than 5×10^8, particularly if they originate from cultures containing few *Gal*$^-$ segregants; (2) the number of transducing particles in these lysates is often nearly equal to the number of lambda particles in the lysate (table 6). These lysates will be referred to as HFT (giving a high frequency of transduction).

Transductions with lysates of heterogenotes

Platings of highly diluted HFT lysate with *Lp*^{s} and *Lp*$^+$ bacteria give a number of papillae. The number of papillae obtained with *Lp*^{s} cells is, however, less than that obtained with *Lp*$^+$. The lower yield with *Lp*^{s} recipients may result at least in part from the loss of potential transductions through lysis of the recipient cell or of some of its early progeny.

With HFT lysates it is possible to transform a large fraction of a cell population, and to observe transduction without strong selection. By adsorbing HFT lambda onto cells, diluting and plating on EMB galactose to obtain well isolated colonies

TABLE 5

Frequency of segregation from the heterogenotes

Heterogenote	Test clones*			Probability of segregation per 10^3 bacterial divisions†
	Number of cells in inoculum	Number of *Gal*$^-$ cells	Total cells	
Gal$_1^-$_//*Gal*$^+$	2.1‡	6	1169	1
		3	595	1
		4	251	4
		23	1252	3.6
		9	1113	2
		19	897	4.3
		103	2750	6.6
		319	1622	36.8
		22	1966	2.0
		0	237	0
Gal$_2^-$_//*Gal*$^+$	1.5§	11	323	8.1
		2	176	3
		8	1669	0.9
		3	317	2
		52	1236	8.2
		0	10	0
		36	1055	6.7
		3	299	2
		6	386	4
		55	1965	5.1

* A fully grown culture in penassay broth was diluted to give about 10 cells per ml. Twenty samples of 0.1 ml were taken up in 0.2 ml serological pipettes which were supported in a horizontal position on a tray. The pipettes were incubated at 37°C for 4.5 hours. Each pipette was then blown out on to an EMB galactose agar plate, and the inside of the pipette washed with 0.1 ml of broth. The washing was added to the plate, and the inoculum spread with a glass rod. After 18 hours incubation at 37°C the number of *Gal*$^+$ and *Gal*$^-$ colonies on the plates was determined.

† Using the equation $a = 0.602r/N \log N$, (modified for the indicated units from LURIA and DELBRÜCK 1943) where r = the number of *Gal*$^-$ segregants and N = the clone size. The probability of segregation is also estimated by the fraction of cultures containing no segregants.

$$a = \frac{2.3}{N} \log \frac{1}{P_0} \quad (P_0 = \text{fraction of cultures with no segregants.})$$

In the first experiment, using $N = 2^{10}$

$$a = \frac{2.3}{1024} \log 1/1/19 = 2.8 \times 10^{-3}$$

In the second experiment, using $N = 2^{10}$

$$a = \frac{2.3}{1024} \log 1/1/11 = 2.6 \times 10^{-3}$$

‡ The assay plates showed this culture to have *Gal*$^+$: *Gal*$^-$ in the ratio 106:4. Of the twenty samples in this experiment, one contained only *Gal*$^+$, one contained only *Gal*$^-$, and 18, both *Gal*$^+$ and *Gal*$^-$. Only the plates that were counted are given. Nine plates were too crowded to be counted accurately.

§ The ratio of *Gal*$^+$:*Gal*$^-$ in the parent culture was 128:19. The twenty cultures were distributed as follows: failed to grow, 9; contained only *Gal*$^+$, 1; contained both *Gal*$^+$ and *Gal*$^-$, 10. One plate had approximately equal numbers of *Gal*$^+$ and *Gal*$^-$ and was assumed to have come from a mixed inoculum.

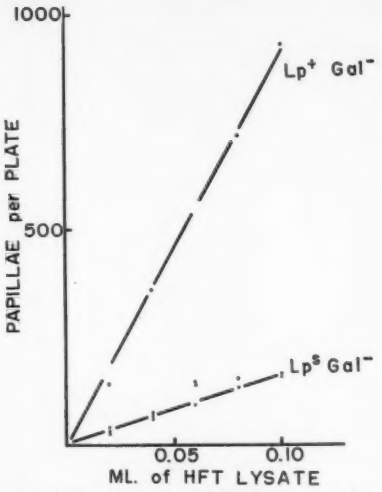

FIGURE 4.—Proportionality between amount of HFT lysate and number of papillae formed from Lp^s and Lp^+ Gal^- cultures. For the assays, a lysate containing 1.6×10^8 phage/ml was diluted a thousandfold.

it is possible to study individual transduction clones derived from single particle infections of isolated bacteria. At the optimal ratio of about 10 lambda particles per cell, the fraction of cells transformed ranged from 5 to 15 percent.

Evidence that lambda is the vector of transduction

That lambda is the vector of Gal transduction is suggested by previous experiments: (1) the 100 percent lysogenization of Lp^s recipients by LFT lysate transductions; (2) the incidence of lysogenicity in transduction to Lp^r recipients. In

TABLE 6

The high frequency of transduction (HFT) given by lysates of heterogenotes

Heterogenote	Lysate of the heterogenote		
	Lambda particles per ml	Transductions per ml	Transductions per lambda particle
Gal_1^-_//Gal^+	1.2×10^8	2.1×10^7	1/5.7
Gal_1^-_//Gal^+	5.8×10^8	1.8×10^7	1/32*
Gal_2^-_//Gal^+	5.4×10^7	3.6×10^7	1/1.5
Gal_2^-_//Gal^+	7.6×10^7	4.2×10^7	1/1.8
Gal_4^-_//Gal^+	1.5×10^8	7.4×10^7	1/2.0
Gal_4^-_//Gal^+	7.3×10^8	2.5×10^7	1/29*

* With the exception of these cases, the cultures used for making the lysates were started from a single apparently pure Gal^+ colony on EMB galactose. The lower ratio in the exceptional cases, and the higher lambda titer is probably the result of the presence in the source cultures of a larger number of Gal^- segregants. Assay of the transductions was made with Lp^+ cells.

TABLE 7

Failure of transduction to lambda-2 resistant mutants

Recipient cells (Lp^+)	Lambda-2 reaction*	Number of fermenting clones	
		No lysate added	0.1 ml of Gal^+-lysate
Gal_1^-	sensitive	1	426 (LFT)
	resistant	1	2
Gal_2^-	sensitive	20	356
	resistant	14	14
Gal_4^-	sensitive	89	296
	resistant	50	57
Gal_1^-	sensitive	2	10^7 (HFT)
	resistant	3	4

* Lambda-2 resistant mutants do not adsorb lambda or lambda-2.

addition, lambda and the transducing agent are adsorbed to about the same degree by Lp^s cells, and both are inactivated by crude anti-lambda serum. More definite evidence was the failure of lambda-2 resistant cells to adsorb either lambda or transducing activity or to be transformed even by HFT lysates (table 7).

Conclusive evidence that lambda is the vector of transduction is found from the behavior of single transduction clones: (1) Heterogenotes formed from HFT lysate and Lp^s cells at low lambda multiplicity are always either overtly lysogenic (Lp^+) or carry a defective prophage (Lp^r) (table 8). (2) Proportionality between number of transductions and amount of lysate at high dilution (fig. 4). For a two-factor-system to be invoked at these dilutions, the accessory factor would have to exceed the lambda by at least 10^{10}, which would imply a concentration of this fancied element in undiluted HFT lysate of 10^{18} per ml, which should be compared with Avogadro's number.

Early segregation of Lp and Gal in transduction clones

HFT Gal^+ lambda was mixed with a culture of Gal_4^- bacteria to give 2.6×10^7 lambda and 7×10^8 cells per ml, a multiplicity ratio of 0.04. The suspension was then diluted and plated on EMB Gal to give about 100 cells per plate. After 24 hours incubation, on 7 plates, a total of 8 colonies with Gal^+ sectors was noted. Each of these colonies was sectored, with a large Gal^- component. Each colony was restreaked, and 20 to 30 Gal^- reisolated from each line. Of the 8 lines, the Gal^- from 3 gave only Lp^+, from 5 gave mainly Lp^s with a few Lp^+. Ten Gal^+ (heterogenote) colonies were also picked from each line. All of them were Lp^+ and of a total of 297 Gal^- segregants subsequently reisolated from these 60 heterogenotic colonies, all were Lp^+ also. The frequent segregation of Lp^+/Lp^s subclones from lambda-infected Lp^s cells has been noted previously (LEDERBERG and LEDERBERG 1953; LIEB 1953). The correlation of Lp^+ and Gal^+ evidently extended, in the 5/8 clones that segregated both markers, to the early intraclonal progeny. Since the heterogenotes do not continue to segregate Lp^s, these results are economically interpreted on the basis of the multinucleate character of the bacterial cells. The early segregation would represent the separation of unaltered Lp^s Gal^- nuclei from the

TABLE 8

Incidence of lysogenicity in isolated heterogenotes

1. *The transductions*

Gal⁻ cells exposed to:	Number of colonies observed		
	Unaltered *Gal*⁻	With *Gal*⁺	*Gal*⁻ partially lysed
Broth	3280	0	0
HFT lysate*	2801	31	54

2. *Examination of the colonies after exposure to HFT lysate*

Colony type	Number of colonies examined	Number of colonies		
		*Lp*ˢ	*Lp*⁺	*Lp*ʳ
Unaltered *Gal*⁻	31	31	0	0
With *Gal*⁺	26	0	23	3

* One ml of cell suspension (4.1×10^9 cells) was added to one ml of HFT lysate (1.2×10^9 plaques per ml, 3.0×10^8 transducing particles per ml) and the mixture incubated at 37°C for 10 minutes. The cells were then centrifuged down, the supernatant discarded, and the cells resuspended in one ml of broth. The suspension was then diluted and plated on EMB galactose agar. The tube contained 3.5×10^9 cells after HFT lysate exposure. 1.1 percent of exposed cells were transformed, and 1.8×10^7 transductions per ml were accomplished.

nucleus with which the prophage-*Gal*⁺ complex has associated. The segregation of *Gal* and stability of *Lp* in the heterogenotic subclones will be taken in later communications.

The failure to observe transduction with lytic lambda

The experiments described above employed UV induced lysates. That lytic lambda, prepared by the growth of lambda on sensitive cells is incompetent in transduction is evident from the following: (1) lytic lambda failed to augment the number of papillae when added to *Gal*⁻ cells on EMB galactose agar; (2) the occasional *Gal*⁺ clones that were found on plates to which lytic lambda was added were all stable and were presumably spontaneous reversions. The lysates used in these experiments were made by growing induced lambda from a Gal_4^- culture on a *Gal*⁺ culture, and the initial tests of competence of the lysates were made on Gal_4^- cultures. In this way, confusion by "carry-over" of the inoculum phage was avoided. The experiments were executed on a scale that should have detected as little as 3% of the activity per phage of LFT induced lysates.

Failure to observe transduction at loci other than Gal

Attempts with LFT, HFT, or lytic lysates to transduce genes at other loci were unsuccessful.

The unsuccessful tests for transductions of prototrophy to auxotrophic cultures involved: histidine; leucine (two loci); methionine; proline; glycine or serine; tryptophane.

The fermentation markers that were not transduced included: lactose (*Lac*$_1$); maltose (two loci); arabinose (two loci); xylose; glucose.

The attempt to transduce streptomycin resistance to sensitive cells was unsuccessful.

In the *E. coli* compatibility system, failure to transduce the following was noted: (1) by lysates of Hfr cultures, F^+ and F^- recipients to Hfr; and F^- recipients to F^+; (2) by lysates of F^+, F^- recipients to F^+.

The most extensive tests were made on genes at loci known to be linked to the *Gal* series (*Hfr*, histidine; CAVALLI-SFORZA personal communication, and proline), or mutations other than *Gal* (W435, *Lac*$_3^-$, LEDERBERG and LEDERBERG 1953 and some auxotrophs) which had occurred coincidently with changes of Lp^+ cultures to Lp^s.

In considering the transduction of specific loci, interactive effects should be kept in mind. For example, papillae were observed on EMB lactose, arabinose, and xylose, respectively, in tests with multiple marker stocks. When purified, however, these papillae were negative for the indicated sugar, but gave galactose-positive colonies. Historically, transduction papillae were first observed in platings of a treated Gal^- Lac^- culture on EMB lactose. The papillae proved to be Gal^+ Lac^- rather than Gal^- Lac^+. Evidently, all these sugars have slight selective potentials for Gal^+ clones.

Other observations

Most lambda lysates are viscous when first obtained. The viscosity is destroyed: (1) by DNAase, an indication that DNA is the cause of viscosity; (2) spontaneously at a slow rate. Exposure of lambda lysates to DNAase has not affected either transduction or plaque titers.

Transduction of the *Gal* gene is not restricted when either the donor or the recipient culture is (1) a prototroph or any of a variety of auxotrophs; (2) *Hfr*, F^+ or F^-, in any combination. Transduction is controlled (1) by the method of lysate production, and (2) the ability of the recipient cells to adsorb lambda. The only genes transduced are the *Gal* loci.

Gal^- mutants in *E. coli* strains other than K-12 that adsorb lambda can be transformed. As in strain K-12 the transformation does not require that the recipient be sensitive; among the susceptible strains are lambda sensitives, lambda immunes, and host modifiers of K-12 lambda (E. LEDERBERG 1954). However, lambda was incompetent when tested on galactose negative mutants of Salmonella, and transducing Salmonella phage (ZINDER and LEDERBERG 1952) failed to transform *E. coli*.

DISCUSSION

Galactose-negative cultures of *E. coli* are transformed to galactose-positive by certain lysates containing the phage lambda. That this process is genetic transduction by lambda particles is established by the following: (1) Gal_y^- cells are transformed to Gal^+ by lysates of Gal_y^+ cultures but not by Gal_y^-. (2) However, Gal_y^+ obtained by reversion regains its ability to transform the Gal_y^-, which emphasizes

the role of the donor genotype in effective transformation. (3) The transformed positives are unstable, and segregate Gal_y^- and not other galactose types. The various "Gal_y" used for these experiments include Gal_1, Gal_2, Gal_3, Gal_4, Gal_6, Gal_7 and Gal_8. (4) All transduction clones obtained from Lp^s recipients become lysogenic for lambda (either Lp^+ or Lp^r). (5) Transduction is not obtained with cells unable to adsorb lambda.

The contrasting features of the *E. coli*-lambda and the Salmonella systems of transduction are summarized as follows:

Range of genes transduced	*E. coli* K-12 phage lambda only *Gal*	Salmonella phage PLT22 any selectable marker
Localization of prophage	*Lp* locus linked to *Gal*	Unknown
Competence of lytic phage	No	yes
Transduction clones	unstable heterogenotes	stable
Efficiency of transduction, per phage	LFT 10^{-6} HFT 10^{-1}	10^{-5}–10^{-6}
Sexual fertility of the host	Fertile, subject to *F* compatibility system	Unknown

The two systems are alike in the following respects: (1) Genetic factors are carried by phage particles; (2) The specificity of the transducing particles is determined by the genetic content of the donor bacteria, in contrast to lysogenic conversions (UETAKE, ET AL. 1955); (3) The genetic material is inaccessible to DNAase and other enzymes; (4) Transduction occurs without regard (except for quantitative changes in yield) to the lysogenic or sensitive status of the recipient cells. In both systems UV induced phage is competent, but lytic phage is competent only in Salmonella.

However, the two systems evidently do not cross-react; lambda does not transform Salmonella and conversely, probably because of the specificity of phage adsorption.

Several of these features may be related in origin. For example, the limitation both on the mode of inclusion in the phage (i.e., only after induction of a lysogenic bacterium), and on the genetic material that can be transduced suggest that the physical proximity of the *Gal* loci to the prophage site determines transduction competence of lambda. This is supported by the linkage observed in crosses of *Lp* to *Gal*. Presumably the linked *Gal* genes may sometimes accompany the prophage into the maturing lambda particle when lysogenic bacteria are irradiated. The failure to obtain lambda particles with transducing activity when the phage is grown lytically on sensitive cells would be explained on this hypothesis, since the lambda may have no specific association with the *Lp-Gal* chromosomal segment during lytic growth.

The heterogenotic clones which result from transduction are isolated through the effectiveness of the *Gal* genes that accompany the prophage. In LFT transductions, this is a rare event; the HFT quality of lysates from heterogenotes may result in part from the prior selection of an effective fragment and its reproduction as such in the growth of the clone.

The persistence of the fragment in transduction clones requires an *ad hoc* explanation, possibly related to the presence of an *Lp* region in the fragment. For example, *Lp* might be closely linked to a centromere; it may function as a centromere itself; it may be adapted to synapse with the homologous site of an intact chromosome.

At any rate, the *Lp* region is singular in at least two respects: it is close to a regular point of breakage in crosses determined by *F* polarity (LEDERBERG and LEDERBERG 1953; NELSON and LEDERBERG 1954; CAVALLI-SFORZA and JINKS 1956) and the *Lp* segment (considered as prophage) is capable of independent replication as a phage. If comparable singular regions exist in Salmonella, they have not yet been revealed in the occurrence of heterogenotes.

The occurrence of sexual recombination and transduction in the same organism raises the technical question of their experimental confusion. Since sexual recombination requires intact cells, and transduction is accomplished with a cell-free lysate, sexual recombination can have no direct bearing on transduction experiments. Furthermore, although crossing is completely blocked between F^- cultures, the compatibility status has no effect on transduction. On the other hand, the rarity of LFT transduction makes it a priori unlikely that transduction will significantly interfere with segregation ratios in crosses.

Crosses of the various combinations of cultures carrying different *Lp* alleles will be presented in detail in further reports. However, they have indicated that combinations involving Lp^+ (where transduction could occur) do not give appreciably different frequencies of Gal^+ than $Lp^s \times Lp^s$ crosses (where lambda transduction is not possible). In addition, the Gal^+ prototrophic recombinants obtained are stable for galactose fermentation. Even crosses of known heterogenotes (capable of HFT lambda) have not given increased frequencies of Gal^+. These observations suggest that lambda transduction has not significantly affected results obtained by crossing.

The mosaic colonies of heterogenotic cultures (fig. 3) are reminiscent of those formed by segregating diploids of *E. coli* K-12. The latter, of course, are segregating blocks of many linked markers, not merely the *Gal* genes. Diploids are, however, more difficult to maintain without the benefit of balanced selective markers. They segregate twenty times as frequently as heterogenotes, as can be judged from the appearance of the colonies and from rates calculated from cell pedigrees (ZELLE and LEDERBERG 1952 and unpublished).

Further studies involving the use of two or more *Gal* markers, and relating transduction to sexual recombination analysis will be presented shortly, together with further consideration of the genetics of the prophage.

SUMMARY

Transduction of several Gal^+ genes from galactose positive (Gal^+) to galactose negative cells (Gal^-) by the bacteriophage lambda has been demonstrated. The resultant galactose positive clones have been found to be heterozygous for the *Gal* region and have been designated as heterogenotes ($Gal^-//Gal^+$). Segregation and the reappearance of Gal^- from the heterogenotes occurs about once per 10^3 bacterial divisions. The low frequency of lambda particles with *Gal* genes ($1/10^6$) from haploid cultures resembles other transduction systems. However, heterogenotic cultures

produce lysates in which nearly every lambda particle carries *Gal* genes. No other markers have been transduced by lambda, and the competence of lambda in transduction depends upon its production from lysogenic cells, rather than by lytic growth on sensitive bacteria.

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